UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

GENETIC ANALYSIS OF *THEILERIA ORIENTALIS* POPULATION IN CATTLE FOLLOWING A THEILERIOSIS OUTBREAK IN VICTORIA, AUSTRALIA

NÁDIA SORAIA SEGREDO SPIRO CUFOS

DISSERTAÇÃO DE MESTRADO EM MEDICINA VETERINÁRIA

CONSTITUIÇÃO DO JÚRI

Doutora Isabel Maria Soares Pereira da Fonseca de Sampaio

Doutor Robin Beat Gasser

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To my Mother, my Wonder Woman

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Cufos, N., Jabbar, A., Carvalho, L.M., Gasser, R.B. (2012). Mutation scanning-based analysis of *Theileria orientalis* populations in cattle following an outbreak. Electrophoresis 33, 2036–2040

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GENETIC ANALYSIS OF *THEILERIA ORIENTALIS* POPULATIONS IN CATTLE FOLLOWING A THEILERIOSIS OUTBREAK IN VICTORIA, AUSTRALIA

Abstract

Bovine theileriosis is a tick-borne disease caused by one or more haemoprotozoan parasites of the genus *Theileria*. In the past, *Theileria* infection in cattle in Australia was largely asymptomatic and recognized to be associated with *Theileria buffeli*. However, in the recent years, outbreaks of theileriosis have occurred in beef and dairy cattle in subtropical climatic regions (New South Wales) of Australia. There is also one published report of a recent theileriosis outbreak on a beef farm near Seymour in the south-eastern state of Victoria. In order to gain an improved insight into the genetic composition of *Theileria* populations following this outbreak, we undertook herein an integrated PCR-coupled mutation scanning-sequencing-phylogenetic analysis of sequence variation in part of the major piroplasm surface protein (*MPSP*) gene within and among samples from cattle involved in the outbreak. *Theileria* DNA was detected in 89.4% of 94 cattle on the Seymour farm; the genetic analysis showed that the *ikeda* and *chitose* genotypes representing the *Theileria orientalis* complex were detected in 75% and 4.8% of 84 infected cattle, respectively, and that mixed populations of these two genotypes were found in 20.2% of infected cattle. Given unpublished reports of a significant increase in the number of outbreaks in Victoria, future investigations should focus sharply on elucidating the epidemiology of *Theileria* to subvert the economic impact on the cattle industry in this state. Although used here to explore genetic variation within the *T. orientalis* complex in Australia, a mutation scanning-based approach has broad applicability to other species of *Theileria* in other countries.

Keywords: *Theileria orientalis* / Cattle / Major piroplasm surface protein (*MPSP*) gene / Mutation scanning-based analysis / Phylogeny

ANÁLISE GENÉTICA DE POPULAÇÕES DE *THEILERIA ORIENTALIS*, EM BOVINOS, APÓS UM SURTO DE THEILERIOSE EM VITORIA, AUSTRÁLIA

Resumo

A teileriose é uma doença transmitida por carraças e causada por hemoprotozoários pertencentes a uma ou mais espécies do género *Theileria*.

Historicamente, a infecção de gado na Austrália, com este parasita, é considerada assintomática e associada especificamente à espécie *Theileria buffeli.* Contudo, nos últimos anos, surtos de teileriose têm ocorrido tanto em explorações de carne como de leite em regiões de clima subtropical da Austrália (Nova Gales do Sul). Recentemente foi publicado um relatório, correspondente a um surto de teileriose perto de Seymour, Victoria, um estado a sudeste do país.

A fim de obter uma melhor compreensão sobre a composição genética das populações de *Theileria* envolvidas neste surto, foi levado a cabo um sistema de análise integrada de PCR - análise de mutações – sequenciação– filogenia, das variações existentes na sequência de parte do gene codificador da principal proteína de superfície do piroplasma (major piroplasm surface protein – MPSP), dentro e entre diferentes amostras provenientes de animais residentes na exploração envolvida no surto.

O ADN do parasita foi detectado em 89,4% de 94 bovinos testados, na exploração de Seymour e a subsequente análise genética mostrou que os genótipos Ikeda e Chitose, representativos do complexo formado por diferentes estirpes pertencentes à espécie *Theileria orientalis*, foram detectados em 75% e 4,8% de 84 animais infectados, respectivamente, e que populações mistas compostas por ambos os genótipos foram detectadas em 20,2% desses mesmos animais.

Dado que, relatórios não publicados apontam para um aumento significativo do número de surtos de teileriose em Victoria, futuras investigações deverão centrar-se fortemente na elucidação da epidemiologia deste parasita, a fim de avaliar o impacto económico que este poderá ter sobre a indústria bovina neste Estado.

Ademais, apesar de usados neste estudo para explorar a variação genética das populações de *T. orientalis* na Austrália, uma abordagem baseada na análise de mutações tem ampla aplicabilidade para outras espécies de *Theileria* presentes em outros países.

Palavras-chave: *Theileria orientalis* / bovinos / Major Piroplasm Surface Protein (*MPSP*) gene / mutation scanning-based analysis / filogenia

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Abbreviations

bp – base pair

BVD – Bovine Viral Disease

C. – Clostridium

DNA – deoxyribonucleic acid

ECF – East Coast Fever

ELISA – Enzyme Linked Immunosorbent Assay

H. - Haemaphysalis

IFAt – Indirect Fluorescent Antibody test

ITS – Internal Transcribed Spacers

mpsp – major piroplasm surface protein

NSW – New South Wales

PCR – polymerase chain reaction

QLD – Queensland

RBC's – red blood cells

RFLP – Restriction Fragment Length Polymorphism

RLB – Reverse Line Blot

RNA – ribonucleic acid

spp. – species

SSCP – Single-Strand Conformation Polymorphism

T. – Theileria

VIC – Victoria

CHAPTER 1 – UNDERTAKING A TRAINEESHIP IN MELBOURNE UNIVERSITY

The present Thesis is based on the traineeship, implemented in the curriculum of the Integrated Master in Veterinary Medicine of the Faculty of Veterinary Medicine, Technical University of Lisbon, (FVM - UTL), which aims the practical application of the acquired knowledge, during the first 5 years of study, in order to prepare the future veterinarian for the labor market, as well as, enable the same with some orientation to the area that most appeals to him/her, since this is a profession with many different applications in many different fields.
For this purpose, there was a chance of a nine months traineeship, from 15th July 2011 to 15th April 2012, at the University of Melbourne in the state of Victoria, Australia.
The University of Melbourne was founded in 1854, the second in the country, by four talented professors from four different schools: WP Wilson (Mathematics), Henry E Rowe (Classics and Ancient History), Frederick McCoy (Natural Sciences) and WE Hearn (Modern History, Literature and Political Economy) (The University of Melbourne, 2012). Currently comprises about 16 schools distributed in seven different campuses (Figure 1), being the main one located in Parkville (Figure 2, 3). It is a prestigious institution, being for a few years in the top 20 of world's best universities, and recognized as a pioneer in research, integrating about 40,000 students from over 120 countries (The University of Melbourne, 2012).
The Faculty of Veterinary Science, it was the first Veterinary school in the country and with more than 100 years of history, has as its mission the training of world-class professionals in veterinary field, and is divided into two campuses: Parkville, in the northern-center of the Melbourne city (Figure 4) and Werribee, in the suburbs (The University of Melbourne, Faculty of Veterinary Science 2012).

Figure 1 – Map with different locations of different campuses of university of Melbourne (adapted from The University of Melbourne, http://brand.unimelb.edu.au/global/contact-maps.html)

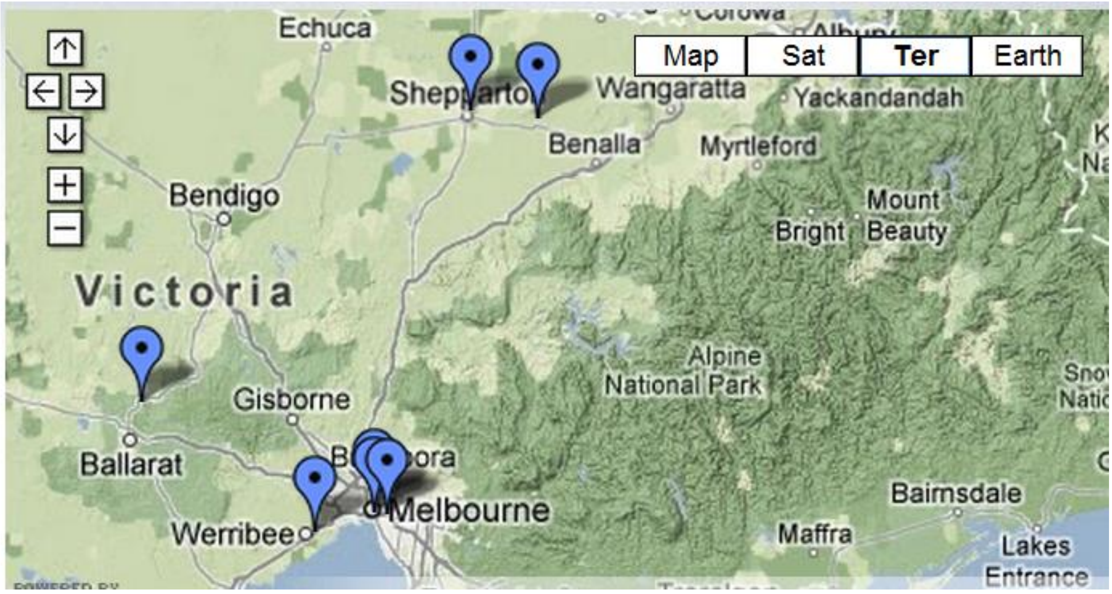


Figure 2 – A view from University of Melbourne, Parkville campus

Figure 3 – Melbourne University Parkville campus map (adapted from The University of Melbourne, http://graduation.unimelb.edu.au/__data/assets/pdf_file/0020/530606/Online_map_-_reduced.pdf

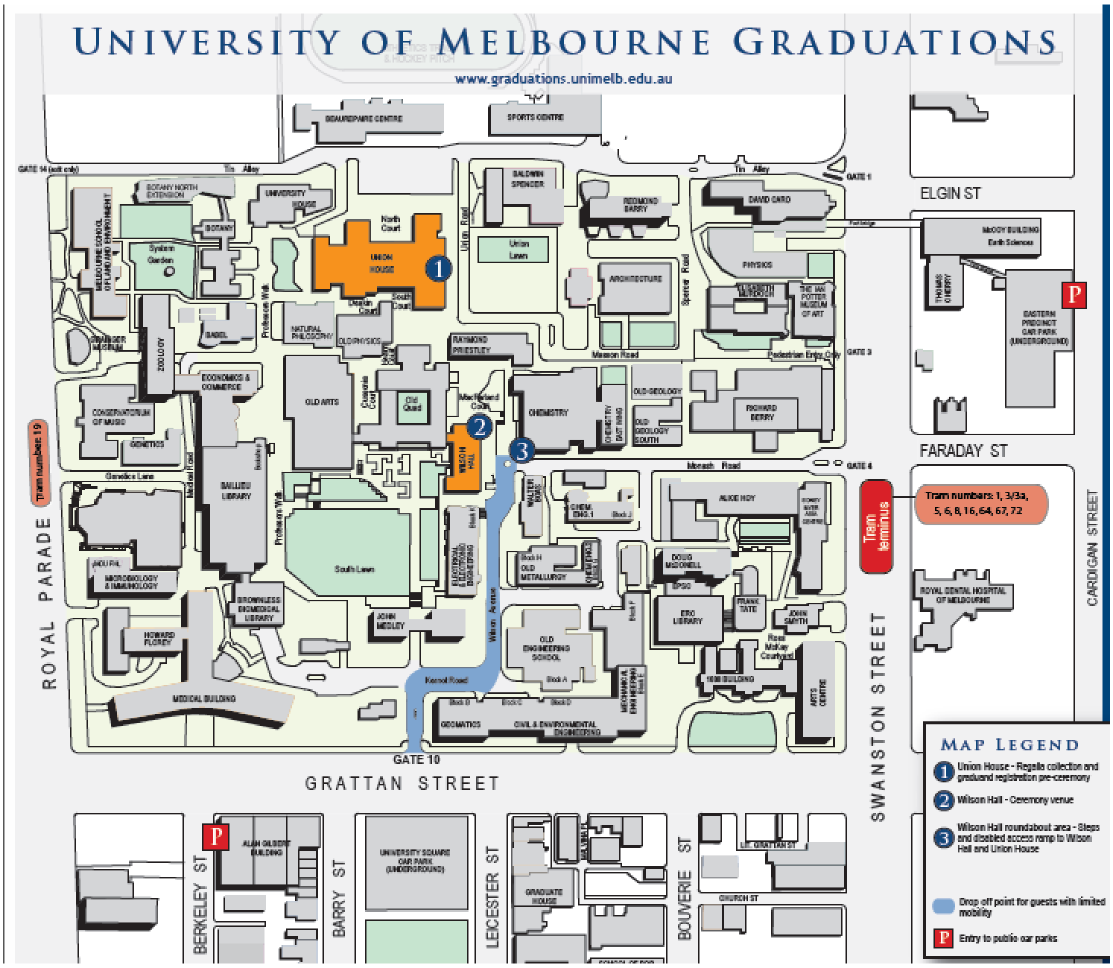


Figure 4 – Veterinary Science Faculty, Parkville campus building

Beyond a focus on training new veterinarian practioners, it also has a large responsibility in different research areas, such as animal production, performance and welfare, infectious diseases, public health, biosecurity, cell biology and morphology, animal biotechnology and small animal medicine.

I had the pleasure to join the Parasite Genetics and Genomics laboratory (Figure 5), directed by the world renowned Professor Robin Beat Gasser, which main goals are "the study of parasites with socio-economic impact in order to reveal their molecular biology to enable further development of new and improved diagnostic techniques, control methods and treatment" (The University of Melbourne, Faculty of Veterinary Science, 2012). The same is composed of a diverse team which includes lecturers, post-doctoral scientists, research assistants and post graduate students, all working together to obtain better results. From the different projects undertaken by this laboratory, stand out transcription projects of different Platyhelminthes, such as *Fasciola hepatica*, and *Haemonchus contortus*; Nematode diagnosis in small ruminants; *Theileria* diagnosis and support on control; and projects in *Cryptosporidium* spp. and *Giardia* spp. in conjunction with Melbourne Water Corporation.

Figure 5 – Gasser's Laboratory structures (original)

As part of the internship, based on the investigation of a recent outbreak of theileriosis in the state of Victoria, we were able to learn new techniques, engage in the daily routine of laboratory work, as well as develop and refine a critical spirit, which opened doors to research world, an unknown field until now, and from which much knowledge and understanding was gathered.

Obviously, at the arrival to the laboratory we were introduced to the entire team to understand the work in which each member was involved. After being explained the specific project for our master's, three weeks were given to get acquainted with the theme of the project and to remember everything that had been learned and that somehow it would be useful in life in the laboratory and to understand thoroughly all techniques (why, how and when to use/apply them).

The second stage of my internship consisted in the laboratory work, during which I was allowed to learn and apply the following techniques:

1. Extraction and purification of DNA for further analysis - learn different techniques for the extraction of genomic material from tissue (ticks and round worms) and blood (cattle), with different kits and protocols, as well as optimization of the method in different situations;

2. Preparation, development and identification of PCR products - learning how to prepare samples and how to assemble the PCR tubes, as well as, handling the different machinery for this purpose and optimization of the reaction to assemble different situations (optimal concentration of reagents, and various stages of the reaction). At this stage I got my first "contact" with bioinformatic tools for primer design;
3. Scanning and analysis of mutations - learning techniques that allow the analysis of mutations within and among different samples, such as SSCP and RFLP;
4. Cloning techniques – preparation of PCR products for further cloning techniques with pGEM T easy vector® (with or without the white/blue selection method) and further analysis of the results (extraction of DNA from clones for posterior sequencing analysis);
5. Sequencing of samples - learning sample preparation for automated sequencing on an independent laboratory, and analysis of results with different bioinformatics tools;
6. Phylogenetic analysis - using different bioinformatic tools that allow an accurate analysis of the results and drawing of phylogenetic relationships among different organisms / populations (Mega5®, Mrbayes®, clustal X®);

Besides all the work done in the laboratory, I was also able to attend different conferences and be aware of other surveys conducted in different laboratories and departments of the college, as well as a short guide tour to the farm affected with the Theileriosis outbreak, in order to collect some information from the responsible veterinarian.

It was surely a unique experience that could not otherwise be possible if not within a traineeship.

CHAPTER 2 – LITERATURE REVIEW

2.1 Introduction

Infectious diseases are of major importance as they can cause substantial damage to humans, animals and plants. Infectious agents include different organisms such as prions, viruses, bacteria, fungi and also protozoan and metazoan parasites.

The metazoan parasites are divided into arthropods (tick, mites, flies and fleas – characterized by their exoskeleton, joined limbs and segmented body) and worms. The latter group includes round worms (Nematoda) and flat worms (Platyhelminthes: Trematoda and Cestoda) (Taylor, Coop & Wall, 2007)

Protozoan parasites are unicellular organisms and comprise 4 main groups, amoeba, flagellates, ciliates and apicomplexans. The Apicomplexa are characterized by the presence of an apical complex, as well by schizogony and merogony stages in their life cycle and include coccidia, haemosporidia and piroplasmidia such as *Theileria* (*T.*) species (Taylor *et al.*, 2007)

Theileria parasites are responsible for causing clinical and subclinical infection, known as theileriosis, an important disease of (mainly) livestock throughout the world and characterized by lymphocytic proliferation and anaemia that can lead to death or, in less severe cases, to a decreased productivity. The most important species are *T. parva*, the causative agent of African East Coast Fever (ECF), and *T. annulata* responsible for tropical theileriosis affecting animals in the Mediterranean basin, middle East and Indo-china (Radostits, Done & Blood, 2007).

In Australia, theileriosis is caused by the *T. orientalis/buffeli/sergenti* group whose classification is still controversial. They are sometimes thought to be benign (in terms of disease), but in Japan, Korea and some parts of China members of this group have a major economic impact in cattle (Kakuda *et al.*, 1998). Parasites belonging to this group are now having an increased impact in Australia. In 2010, Izzo, Poe, Horadagoda, De Vos & House reported a small number of haemolytic disease outbreaks in New South Wales (NSW) associated with *T. orientalis/buffeli/sergenti* group infection, mainly in peri parturient cattle moved from coastal areas to inland farms. In early 2011, there was a report of a similar outbreak in Victoria (VIC) (Islam, Jabbar, Campbell, Cantacessi & Gasser, 2011) a state in which theileriosis disease was never recorded before. Following this report, there has been a series of new outbreaks in this particular state of Australia (Dr. Michael Jeffers, Department of Primary Industries [DPI], Victoria, personal communication; January 2012).

2.2 Literature Review aims

The present thesis investigates, the genetic composition of *Theileria* populations infecting cattle on one particular farm suffering from the initial outbreak in Victoria. In order to provide a foundation for the study it was essential to first review and critically appraise the literature, establish the current state of knowledge and then to identify all the gaps in the knowledge and understanding of this disease globally, in Australia and in Victoria. Therefore, the aims of this chapter were to explore some areas, including the Australian beef production system, taxonomy and classification of the parasite, as well as, life cycle, pathogenesis, epidemiology and aspects of treatment, control and diagnosis and then formulate the specific aims of the thesis.

2.3 Beef production in Australia

Australia is one of the largest exporters of beef in the world (Australian Red Meat, 2012), and exports more than 60% of its entire herd, mainly for the United States and Japan (The Australian beef industry – the basics at www.pwc.com.au).

To this fact contributes not only its extensive territory, as well as the fact that most of the restrictive diseases to cattle production are absent in Australian farms, which puts their meat on top of the safest, and therefore most sought after (Australian Red Meat, 2012).

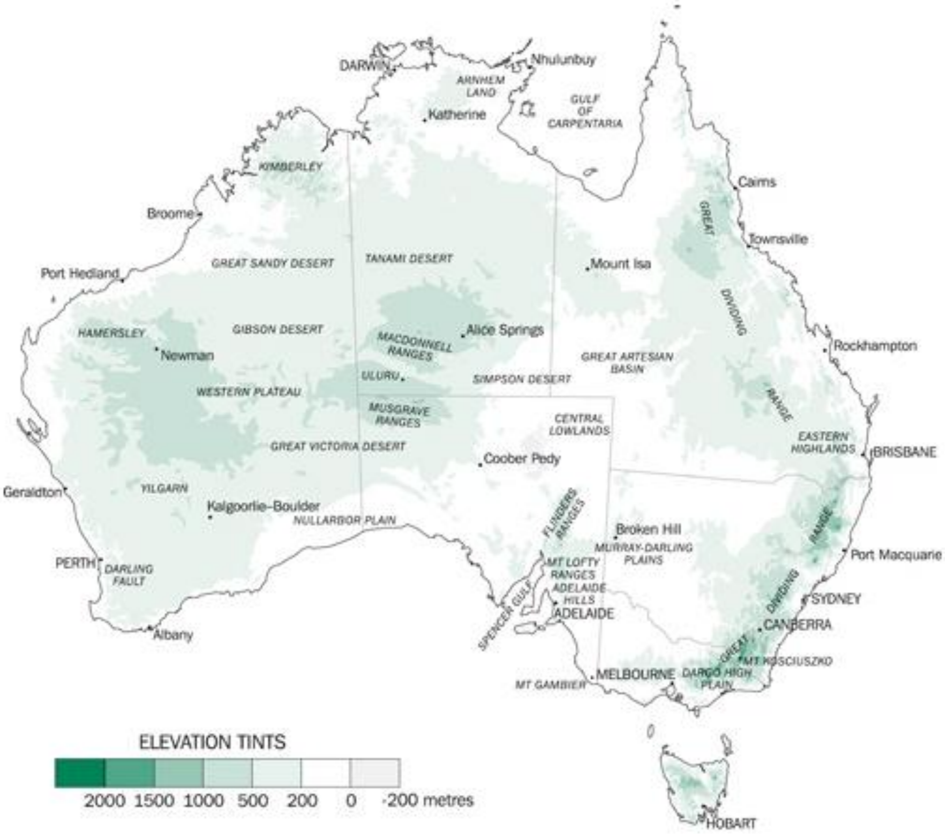
Listed below are some factors that contribute to this success.

2.3.1 Geography and climate in Australia

The Australian territory has approximately 7.7 million km^2 and is divided into seven states: Northern Territory, Queensland, New South Wales, Victoria, Western Australia, South Australia and Tasmania to which are added up to 12,000 islands. Its location in coordinates is: latitude, between 10°41´ south (Queensland) and 43°38´ south (Tasmania) and longitude between 113°09´ east (Western Australia) and 153°38´ east (New South Wales).

It is the second flattest and driest country-continent, right after Antarctica, and many of its landscape are dated to millions of years. It is possible to observe their different elevations in the map in Figure 6 and one should draw attention that mountainous areas, i.e. landscape above 2000m, are concentrated in a small region of NSW (The Australian Bureau of Statistics, 2012).

Figure 6 – Australian map, showing the different elevation landscape (adapted from The Australian Bureau of Statistics, http://www.abs.gov.au)



It is without doubt one of the countries with the greatest variety of climates, that creak from the tropical north and temperate south, passing through the more arid climates in the most interior areas of continental plate. For the same reason fluctuations in temperature and rainfall may be large. By way of example, at north, temperatures are more or less constant throughout the year, having rainfall season from November to April and a drier season during the remaining six months of the year. Unlike in most southern states, where the variations are more pronounced and therefore the four seasons of the year are well defined (The Australian Bureau of Statistics, 2012).

2.3.2 Beef production system

The cattle settling began on the arrival of the first Europeans in 1788 that brought with them six head of cattle purchased in South Africa. The herds have grown ever since, and in 2005 Australia had 2% of world's bovine population and ranked in 10th place of the largest meat exporters (The Australian Bureau of Statistics, 2012).

Nowadays, the herd consists mainly of two types of breeds:

- *Bos indicus* or tropical breeds (Brahman) (Figure 7), imported from India in 1930s (The Australian Bureau of Statistics, 2012) that comprises mostly cattle from the north as they are adapted to extreme temperature conditions in that region (Australian Red Meat, 2012);
- *Bos taurus* or temperate breeds originating from Great Britain (Hereford and Shorthorn) and the rest of Europe (Limousine, Charolais etc.) (Figure 8). These make up the herds from further southern areas, in rainfall regions. The crossbreeds from these animals are used mainly for later finishing as they give larger animals for target markets (Australian red meat, 2012).

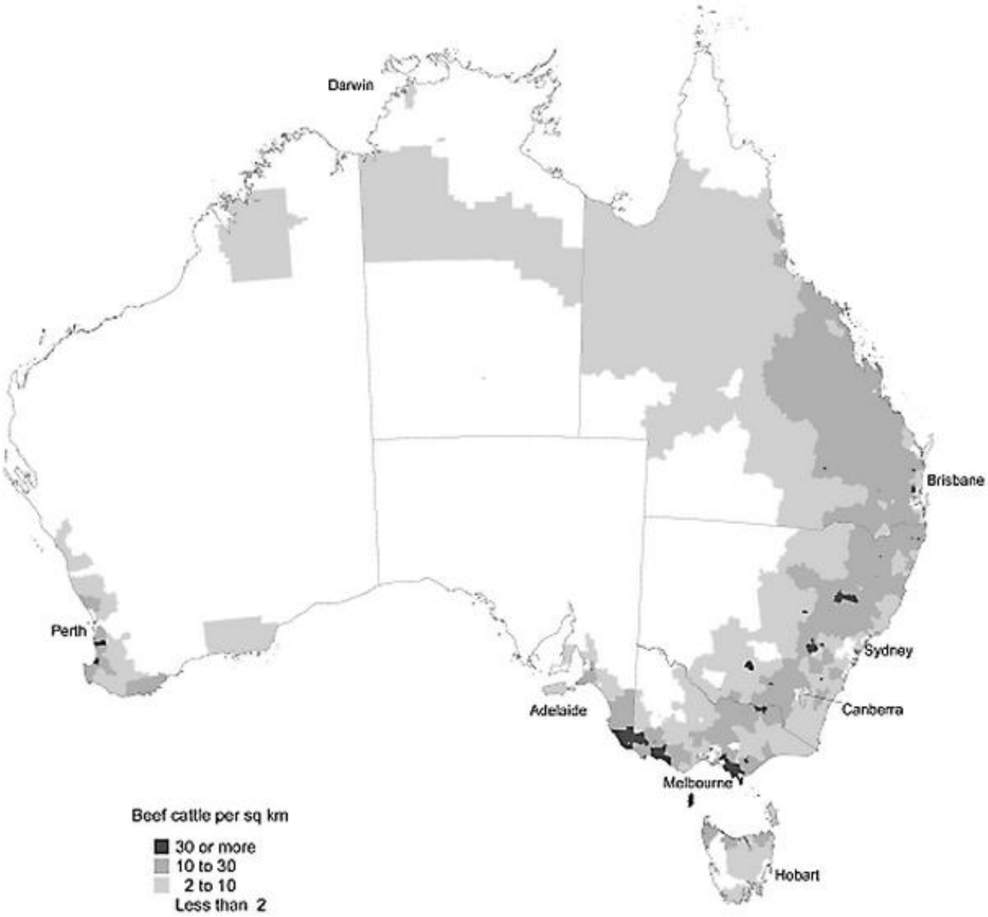
Figure 7 – Brahman breed (original from http://www.ansi.okstate.edu/breeds/cattle)

Figure 8 – Temperate breeds (adapted from http://www.ansi.okstate.edu/breeds/cattle)

As breeds adapt to different Australian climates, also production systems vary, depending on the region where we are.

In northern states such as Northern Territory, northern Queensland, and Western Australia, there is a more extensive system of production, with grazing cattle, and low population densities. Whereas in the southern states the holdings are maintained in an intensive system of small farms where animals are fed with introduced pastures and fodder crops (Figure 9) (Australian Red Meat, 2012).

Figure 9 – Beef cattle distribution in Australian territory in 2001 (adapted from The Australian Bureau of Statistics, http://www.abs.gov.au)



2.3.3 Beef industry

The Australian beef industry constitutes the majority of agricultural industry. In 2005 from 133,000 farming establishments, 33250 were destined to growing cattle for beef industry, which represents almost 27% of all agricultural house holdings (The Australian Bureau of Statistics, 2012).

Most of the beef produced in Northern regions, extensively, is sold to United States, as livestock for other countries, or goes to feed-lot properties, while the cattle produced in south is destined to Japanese and Australian market.

The feedlot system was introduced in Australia in late 1950's, but only during the 1980's it started developing and gain more supporters (Australian Red Meat, 2012). Usually it receives grazing cattle for late finishing on highly energy grain diets for 30 to 300 days depending on the target market (fewer days for national consumption and more days for Japanese and American market).

2.4 Classification and background

Theileriosis is an important tick-borne disease caused by heteroxen haemoparasites of the family Theileriidae (order Piroplasmidia, class Sporozoa and phylum Apicomplexa) (Figure 10).

Nowadays, five species are known to infect cattle worldwide, causing different syndromes, which are characterized from severe clinical disease to mild or non-pathogenic infection (Figure 11 and Table 1) (Radostits *et al.*, 2007).

Figure 10 - *Theileria* genus classification (adapted from Navarrete *et al.*, in Parasitologia Veterinaria, 2002)

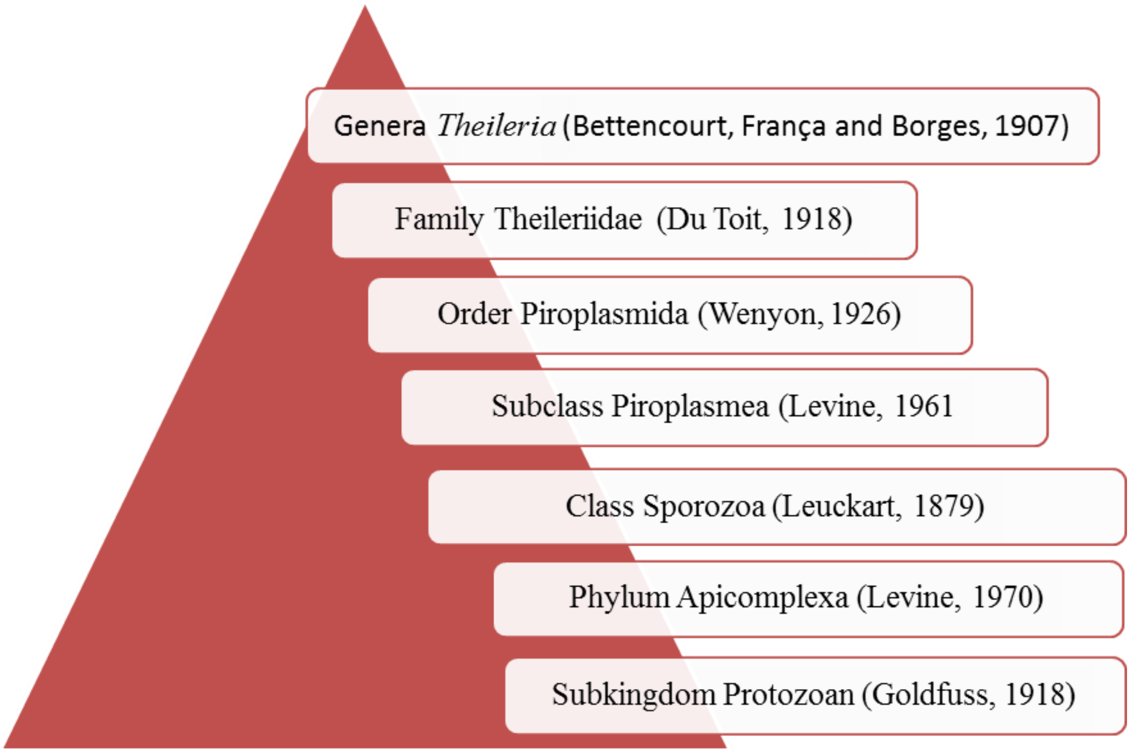


Figure 11 – Most important *Theileria* species distribution (adapted from Welcome Trust Project for Tropical theileriosis, 2007, at http://www.theileria.org)

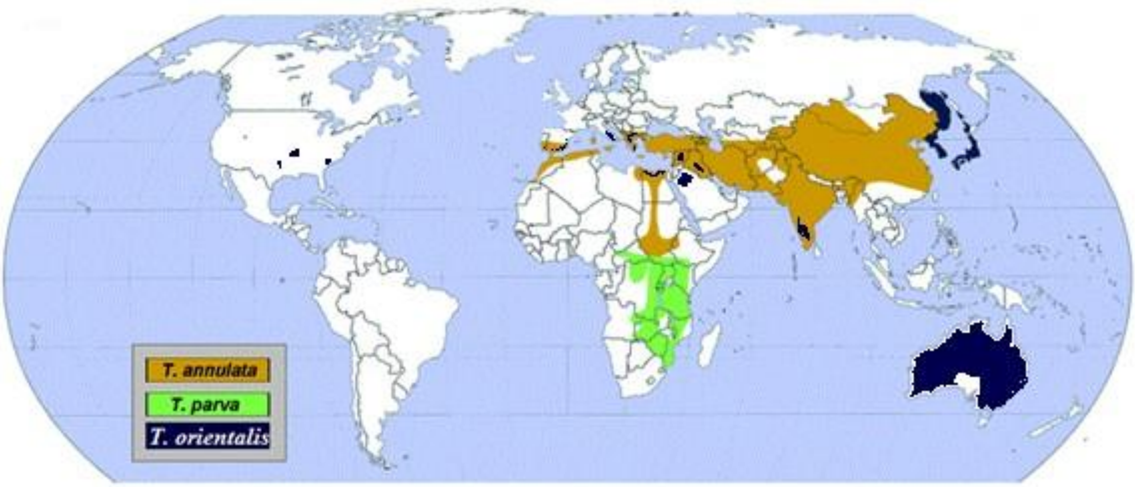


Table 1 – Most important *Theileria* species affecting cattle, their vector, distribution and pathogenicity (based on Radostits *et al*. 2007)

Species	**Vector**	**Distribution**	**Syndrome**	**Pathogenicity**
T. parva	*Rhipicephalus appendiculatus*	East and Central Africa	ECF Corridor disease January disease	High
T. annulata	*Hyalomma anatolicum*, *H. dentritum*	Mediterranean countries Middle East India China	Tropical theileriosis	High
T. orientalis	*Haemaphisalys longicornis*	Southern Europe Middle east Asia Australia	Oriental theileriosis	Mild
T. mutans	*Amblyomma* sp.	Africa Caribbean Islands	-	Low
T. taurotragi	*Rhipicephalus* sp.	Africa	Cerebral theileriosis/ “turnin sickness”	Low

Theileria organisms parasite mononuclear phagocyte system and the species considered with most impact on livestock production, also known as lymphoproliferative species (Dobbelaere & McKeever, 2002), are *T. parva*, responsible for different syndromes in East and central Africa, and *T. annulata* which infects cattle, in the Mediterranean basin, Middle East and Indo-China. These parasites are highly pathogenic due to their schizonts pathogenicity, which induce, along with their own multiplication, uncontrolled proliferation of their host cells. In other words, each infected lymphoid cell that divides originate two daughter-cells, both infected, resulting in the exponential multiplication of the parasite in a short period of time.

Although these species have this point in common, *T. parva* and *T. annulata* differ in some aspects.

T. parva affects cattle from countries in Africa and is transmitted by *Rhipicephalus* spp. Its main reservoir is the African buffalo, and the parasite is responsible for three different syndromes: East coast fever, transmitted by ticks feeding on infected cattle and transferring it to susceptible animals. In enzootic areas the mortality rate may exceed 90% (Mehlhorn, 2001), and recovered animals may have their productivity affected by several months. It was eradicated from southern Africa by restrictive control and slaughter measures; Corridor disease, transmitted from infected buffalo to cattle, is similar to the former syndrome, however the course of the disease is faster, and death occurs within days. Recovered animals stay infected for life; January disease occurs in Zimbabwe and appeared after eradication of ECF. It is closely related to activity peak of the tick vector and therefore its name. As for the first syndrome its transmission is from cattle to cattle, and symptomatology and epidemiology are the same.

T. annulata is the causative agent of tropical theileriosis, has a wider distribution than *T. parva* and is transmitted by ticks of the genus *Hyalomma*. Navarrete, Serrano & Reina et al. (2002), estimates that more than 250 million animals are infected by this agent, which parasites both, cattle and buffalo. Although the mortality rate is slightly lower than in ECF (around 70% in enzootic areas), it represents a huge problem, mainly for small producers, since its control is costly.

Despite of the fact that initial symptoms of *T. annulata* infection resemble the ones caused by *T. parva*, such as general lymphadenopathy and fever, in more advanced stages, animals show up with symptoms of pronounced haemolytic anaemia and jaundice, most probably caused by their intraerythrocitic stage.

More benign species, the non-lymphoproliferative group is composed by *T. orientalis, T. mutans* and *T. taurotragi,* whose impact is not that important due to their low pathogenicity. The mortality rate is estimated to be up to 30% in introduced and exotic cattle (Irwin, 2012).

Moreover, *Theileria* spp. can produce nervous symptomatology recognized as turning sickness disease, when schizonts build up in capillaries of the brain. The cattle present circling movements, incoordination of the hind legs, loss of orientation, ataxia, opisthotonus and paralysis (Capucchio *et al.,* 2011).

In Australia theileriosis is thought to have entered the country by introduced goods from Japan infested with the tick vector (Riek, 1982). It is caused by members of the *Theileria orientalis/buffeli/sergenti* group, which presently comprises eight different genotypes based on the major piroplasm surface protein (MPSP) gene: 1- chitose, 2- ikeda, 3- buffeli and genotypes 4 to 8, (Ota *et al*., 2009; Kamau *et al*., 2011). The classification of *Theileria* spp. is based mainly on morphology, presence of schizonts, geographical origin, pathogenicity, mammalian and tick preferences, and/or serology. As these criteria have usually proved unreliable, in recent years, molecular markers have been used to provide a better insight into their classification (Gubbels *et al*., 2000). Despite this fact the nomenclature/classification of the *T. orientalis/buffeli/sergenti* group is not yet clear, with different studies adopting different names: *T. sergenti* in Japan, *T. buffeli* in Australia and *T. orientalis* elsewhere (Fujisaki, 1992).

Some researchers believe that all genotypes of the group belong to a single species, while others believe that the group comprises different, but closely related species (Kawazu, Sugimoto, Kamio & Fujisaki, 1992; Fujisaki, Kawazu & Kamio, 1994; Stewart, Uilenberg & deVos, 1996). For a better understanding of the evolution of *T. orientalis/buffeli/sergenti* classification, a summary is given below (Table 2).

Table 2 – Chronology of different studies which attempted to classify members of the *Theileria sergenti/buffeli/orientalis* complex

Year	Researcher	Achievement
1906	Theiler	Described *T. mutans* for the first time;
1908	Schein	Isolated a theileria from the water buffalo, in Vietnam, and called it *T.buffeli*;
1910	Seddon	Recorded a *Theileria* parasite in Australian cattle and name it *T. mutans;*
1912	Neveu-Lemaire	Isolated a *Theileria* parasite from Asian water buffalo in Southeast Asia and named it *T. buffeli*;
1923	Schein	Failed transmission of the parasite from cattle to buffalo and vice versa;
1926	Wenyon	Named sheep's *Theileria* as *T. sergenti;*
1930	Yakinnoff Dekhtereff	Described a parasite causing clinical theileriosis in Eastern Siberia and named it *T. sergenti;*
1931	Yakinnoff Soudatschenkoff	Described a similar parasite as the previous in the same area and named it *Theileria orientalis;*
1966	Rogers	Reported 3 fatal cases of infection with *Theileria mutans* in Queensland;
1976	Callow	Showed that *T. buffeli* can be transmitted from buffalo to cattle;
1977	Uilenberg	Stated that Australian *T. mutans* is in fact *T. sergenti* Yakimoff and Dekhtereff 1930;
1981	Morel Uilenberg	Suggested that Australian *Theileria* is either *T. orientalis* (Yakimoff and Dekhtereff 1930) or *T. buffeli* (Neveu-Lemaire 1912);
1984	Callow	Based on *Theileria* transmission from buffalo to cattle state that it should be named *T. buffeli*;
1985	Uilenberg	Concluded, based on serological and morphological characteristics, that strains from Japan, Australia, Iran, Britain and USA were identical, with a more pathogenic strain from Korea, and named them all *T. orientalis*;
1985	Shastri	Failed transmission of *T. buffeli* from buffalo to cattle;
1987	Stewart	Designated Australian strain as *T. bufelli* (Callow 1984) after demonstrating the non-transmissibility with *Haemophysalis longicornis* ticks and suggested *H. brancofti* and *H. humerosa* as the main vectors;
1991	Kawazu	After transmission studies and protein analysis of piroplasm suggested two groups to classify the benign *Theileria* from Japan (*T. sergenti*), Australia and Britain (*T. bufelli/orientalis*);
1991	Sugimoto	Analysed proteins from different isolates in a 2D gel and concluded that *T. buffeli* and *T. orientalis* were identical, with *T. sergenti* showing a completely different spot-pattern suggesting that this parasite may belong to a different species;
1992	Fujisaki	After a review of transmission, phenotypic and genomic experiments, suggested that Japanese *T. sergenti* might be a different species and the Australian, *T. buffeli*, and British, *T. orientalis*, might belong to one and the same species;

Table 2 (cont.) – Chronology of different studies which attempted to classify members of the Theileria sergenti/buffeli/orientalis complex.

1992	Kawazu	They compared the nucleotide sequence of *T. sergenti* and *T. buffeli* for the 33/34kDa proteins and showed high similarity, but when compared by RFLP they showed distinct patterns; Built cDNA libraries for *T. buffeli* and *T. sergenti* and screened them with rabbit anti sera. The proteins 33/34kDa showed species-common and species –specific epitopes;
1994	Fujisaki	Considered *T. bufelli* and *T. orientalis* as the same species and *T. sergenti* as a separated one;
1994	Kubota	Found two major allelic forms for *T. Sergenti*: ikeda and chitose types;
1995	Kubota	They found a new *buffeli* allele in Japanese isolate and named it B2, being the B1 the *buffeli* type from Australian isolates;
1996	Kubota	After compared chitose types sequences from Australian and Japanese isolates concluded that they were 98.5% similar;
1996	Stewart	After a review of different characteristics concluded that there was no valid reason for distinguish more than one species and that they all should be name *T. buffeli*;
1998	Kim	Found parasites with three different MPSP sequences. Demonstrated that among the same type the differences were very small regardless the geographic origin, but different types are very different even when they come from the same isolate;
1999	Kawasu	Based on phylogenetic analysis and vector tick subgenus experiments, proposed the name *T. buffeli* to characterize the parasites of Asian buffalo, *T. orientalis* to benign theilerias of cattle. Divided the latter into two subspecies: *T. orientalis orientalis* and *T. orientalis sergenti*;
1998	Chae	After analysis of the variable region V4 of SSU rRNA, they described 7 genotypes A, B, C, D, E, H and Warwick for cattle;
2000	Gubbels	After a phylogenetic analysis of SSU rRNA and MPSP genes concluded that all known *T. bufelli* isolates must been originated from the buffalo stock and suggested that all should be in the same species, *T. buffeli*, until further studies suggest otherwise subspecies;
2004	Kim	Found a seventh type and divided the species in two sister groups: group 1(type 2 + type 7) and group 2 (type 3 + type 5);
2006	Kim	Re-grouped the species in (type 2+ type 7) (type 3 + type 4);
2009	Jeong	Classified the parasites into 8 different types and the Brisbane isolate (unclassified) divided into 2 sister groups: (type 2 + type 7) (type 3 + Brisbane);
2010	Liu	Reclassified *T. orientalis* MPSP type 6 as *T. sinensis;*
2010	Khukhuu	Found a ninth genotype: N3, and divided into two groups: Ikeda (type 2 + type 7) and Chitose (type 1, 3 ,4 ,5 8 N3)

As shown in the table above, there is still much to be done to reach a consensus regarding the name and number of species that constitute this group, as well as in the knowledge of the disease.

Therefore, in order to simplify the reading, from this point on, the group will be referred to as *T. orientalis*, as the name *T. sergenti* was already used for a sheep's parasite and it is not yet proved that buffalo can be infected with this *Theileria* spp., therefore the name *T. buffeli* is still controversial (Fujisaki, 1992).

2.5 Life cycle and pathogenicity

The life cycle of *T. orientalis* is similar to those of other *Theileria* spp. (Figure 12). The parasite undergoes gametogony (sexual reproduction) and sporogony (asexual reproduction) in the tick vector and schizogony and merogny (asexual reproduction) in the mammalian host (Navarrete *et al*., 2002).

2.5.1 The tick vector

Theileria parasites are transmitted by arachnids belonging to the SubOrder Ixodides, commonly known as ticks. It is divided into two distinct families, the Argasidae family and the Ixodidae family also called as hard ticks.

The latter is extremely important, both in veterinary practice as in public health not only due to the direct damage caused by the tick, through the ingestion of blood, secretion of toxins (as in the tick paralysis disease) and induction of an exacerbated inflammatory reaction, but also due to the fact that different species act as vectors of different diseases caused by viruses, bacteria, and parasites (piroplasms and ricketsias).

They owe their designation of hard ticks, due to the presence of a dorsal shield, complete in the male and partial in the engorged female, and among other characteristics of the family we also highlight the terminal position of the capitulum and pronounced sexual dimorphism of the adult forms.

Cosmopolitan parasites, mostly ubiquitous and heteroxens, comprising numerous genera capable of transmitting different *Theileria* spp., depending in the region of the planet: *Ixodes, Amblyoma, Hyalomma, Boophilus, Rhipicephalus, Dermacentor* and *Haemaphysalis.*

In Australia, currently it is accepted that the species responsible for *T. orientalis* transmission is *Haemaphysalis longicornis*, also known as bush tick, parasitizing, preferably, cattle but also

found in other mammals and sometimes birds. These ticks have a small capitulum and no eyes, being the place of excellence for attachment the ear, neck, shoulders and perineum.
They are 3 host ticks, which means that every different stage will feed in a different host, falling to the ground after the blood meal for moulting.

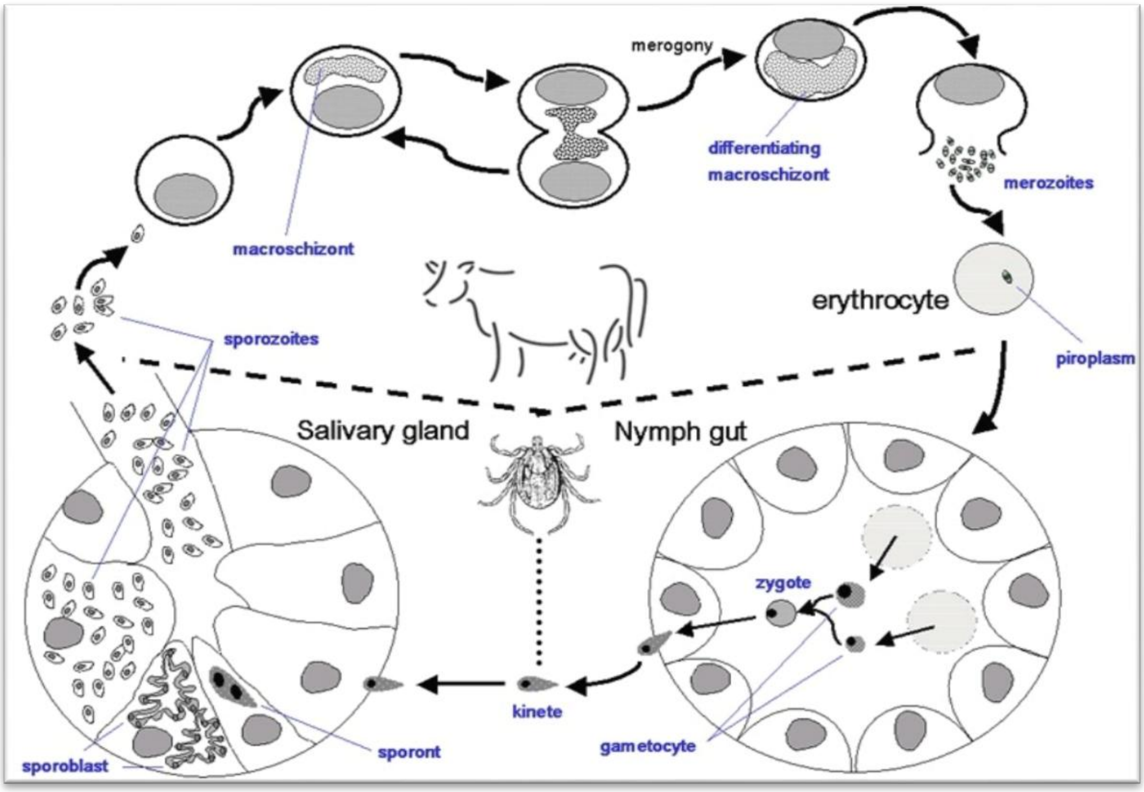
2.5.2 Life cycle in the tick vector

The ixodid tick becomes infected through the ingestion of intraerythrocytic piroplasms when it feeds on an infected animal. In the gut, macro and microgametes merge to give rise to the zygote that develops to an ookinete in order to penetrate the intestinal wall. This mobile body travels via haemolymph, to specialised cells of the salivary glands (Navarrete *et al*., 2002), where it undergoes sporulation, which culminate in the formation of sporoblasts. When the tick feeds in the next animal a new multiplication cycle begins to form sporozoites (infective form to the mammalian host), inoculated along with the saliva of the feeding tick.

2.5.3 Life cycle in the mammalian host

Once injected into the definitive host, the sporozoites infect reticuloendothelial cells and approximately 10 days after inoculation, macro- and microschizonts are found in lymph nodes, spleen and liver. Schizogony is transient for this species, and the parasite is not able to induce uncontrolled multiplication in the host cell: therefore fatal lymphoproliferation, so characteristic of highly pathogenic species, is not observed (Sugimoto & Fujisaki, 2002).
The schizonts, or Koch's blue bodies, differentiate into merozoites which, after rupturing the host cell, invade red blood cells (RBCs) giving raise to piroplasms that will be ingested by the next tick vector, after which, a new cycle begins.

Figure 12 – Life cycle of *Theileria* species (generalised) (adapted from Welcome Trust Project for Tropical theileriosis, 2007, http://www.theileria.org)



2.5.4 Pathogenicity

It is believed that *T. orientalis'* piroplasms are responsible for causing disease, since, at the time they appear in blood stream, anaemia, transient fever and a reduction in white blood cells counts can be observed (Kawazu *et al*., 1991; Sugimoto & Fujisaki, 2002). Although the mechanism responsible for the onset of haemolytic anaemia is still unclear, some published works showed that haemolytic and oxidative mechanisms may be involved in RBCs damage (Hagiwara *et al*., 1995; Shiono *et al*., 2001). This may lead to an increase on the osmotic fragility of the erythrocyte as well as in the appearance of abnormal cells (Yagi, Furuuchi, Takahashi & Koyama, 1989) that will accelerate the removal of parasite as well as non-parasite cells by activated T cells, NK cells and monocytes (Ishii *et al*., 1992). Although anaemia induced by *T. orientalis* is not fatal, in the presence of mixed infections with another haemoparasites, it can lead to death in the absence of treatment.

2.6 Epidemiology

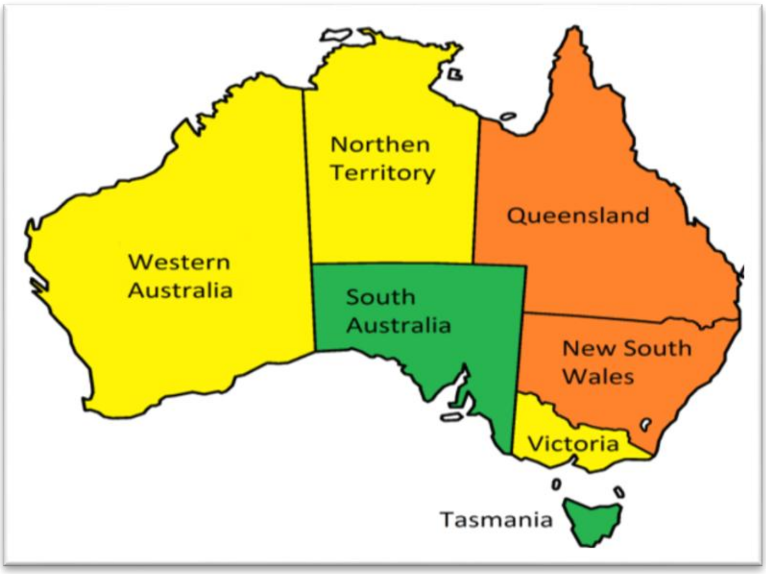
T. orientalis is transmitted by ticks of the genus *Haemaphysalis*, but it is possible that other congeners are implied in its transmission, which may explain its wide distribution (Gubbels *et al.*, 2000). As the tick vector is essential in the parasite life cycle, the distribution of both, *Theileria* and tick, are linked, with seasonal peaks of the tick activity corresponding to an increased infection rate with the parasite (Ota *et al*., 2009).

Transmission is transtadial, i.e., stage to stage in the tick vector (Riek, 1982), iatrogenic, by parasitized blood transfusion or inoculation (Uilenberg, Perie, Spanjer & Franssen, 1985) and Baek *et al*. (2003) demonstrated transplacental transmission, by showing the presence of piroplasms in blood samples of “pre-colostral” calves, and schizonts in spleen smears of aborted foetuses. There are no records of transovarial transmission in the three-host tick till date.

Several studies were carried out and suggested a worldwide distribution of *T. orientalis* from temperate to subtropical zones (Sugimoto & Fujisaki, 2002). The parasite is prevalent in southern Europe countries (Papadopoulos, Brossard & Perie, 1996; Ceci *et al*., 1997; Brigido *et al*., 2004; Garcia-Sanmartin, Nagore, Garcia-Perez, Juste & Hurtado, 2006), Middle East (Uilenberg & Hashemi-Fesharki, 1984; Cicek, Eser & Tandogan, 2009) and Indo-Pacific region (James *et al*., 1984; Luo & Lu, 1997). Although there is little information about the prevalence of *T. orientalis* in America, a fatal case was reported in Missouri (Stockham *et al*. 2000), and the parasite is known in other states such as Texas, North Carolina and Michigan (Cossio-Bayugar, Pillars, Schlater &Holman, 2002).

In Australia, this taxon was first recorded in 1910 and it is thought to have entered the country via goods infested with *Haemaphysalis* ticks from Japan (Seddon, 1966 cited by Riek, 1982). Subsequently the parasite has spread throughout the country, with prevalence recorded from all states except South Australia and Tasmania (Figure 13) (Stewart *et al*., 1996). Highest prevalence is recorded in the eastern parts of the country, namely in New South Wales (NSW) and Queensland (QLD) with animal prevalences of 60% and 41% respectively (Sedon, 1966 cited by Stewart, Standfast, Baldock, Reid & de Vos, 1992).

Figure 13 – Australian map showing *T. orientalis* record in different states. States in green represent those whose presence of the parasite has never been registered. States in yellow represent the states without official/studied prevalence and, finally, the orange states represent those with the highest prevalence recorded to date (original).



Oriental theileriosis has been reported mainly in grazing cattle, which had contact with the parasite early in their life, becoming carriers, after recovery, and a source of infection for ticks, maintaining an endemic status in the herd (Stewart, de Vos, McHardy & Standfast, 1990). Although, in general, it causes subclinical disease, leading to a decrease in daily gain/growth of calves and low productivity in adults (Jeong, Kweon, Kang & Paik, 2003), is known that, under stress conditions such as mixed infections with other pathogens, transportation or environmental alterations, animals may succumb to disease, and death may even occur in more severe cases (Tanaka *et al*., 1993; Izzo *et al*., 2010). In some Asian countries like Japan, Korea and some areas of China it causes clinical disease associated with haemolytic anaemia, causing major economic losses. Recently in Australia there have been reports of outbreaks in NSW and VIC (Izzo *et al*. 2010; Islam *et al.* 2011), and a fatal case was reported from a single cow in Michigan, USA (Cossio-Bayugar *et al*. 2002).

Onuma, Kakuda & Sugimoto (1998) demonstrated that most of these field isolates were associated to the presence of Ikeda type, the most pathogenic strain of *T. orientalis*.

It must be noted that outbreaks occur especially when naive animals are exposed to the parasite, either by cattle movement to endemic areas, or by the introduction of infected ticks into clean areas.

Most of field isolate observed are composed by mixed infection of two or more different types of *T. orientalis,* with rare records of single infection. Thus Matsuba *et al*. (1993) Kubota, Sugimoto & Onuma (1996) and Onuma *et al*. (1998), showed changes in the dominant population during chronic infection or during transmission from cattle to vector or vector to cattle. Both events may show a way of the parasite to evade and disrupt the immune system of the host.

It is however interesting that, a significant level of breed resistance to infection with this parasite has been observed. In Japan, Japanese Black cattle (Terada, Ishida & Yamanaka, 1995) appear to be more resistant to infection than Holstein Frisian breed, and in other studies local breeds showed less or no symptoms of disease when compared with exotic breeds (Liu *et al*., 2010; Aparna *et al*., 2011; Yokoyama *et al*., 2011).

Notwithstanding this can allow the animal to build an immune response against more pathogenic strains, and Gale, Leatch, Dimmock & Gartside (1997) showed that natural infected animals when challenge with *Anaplasma* spp. develop low parasitemia levels compared with free- *Theileria* animals.

2.7 Treatment and control

An early diagnosis, a proper choice of chemotherapy against piroplasms and the adoption of the best prevention method for each particular geographical region are the best way to fight theileriosis.

2.7.1 Chemotherapy

As an intraerythrocytic parasite, *Theileria* is challenging to treat, and researchers have not yet found, a drug, thus far, capable of eliminating infection. Thus, recovered animal usually become carriers for life. Nevertheless, if chemotherapy is given on time, at an early stage, clinical signs can be reduced and the animal can be saved (Stewart *et al*., 1996).

In Australia, only tetracyclines are approved for use against theileriosis. Therapy usually involves the administration of oxytetracycline (10-20 mg/kg, intramuscular, daily), intravascular fluid therapy and blood transfusion (Izzo *et al*., 2010).

However other drugs are known to decrease clinical signs. Aminoquilonine compounds such as primaquine were showed to act against piroplasm stage (Neitz, 1950 and Callow, 1984 cited by Stewart *et al*. 1996). Stewart and de Vos (1990) published two works in which demonstrated that primaquine alone does not work, but in conjunction with buparvaquone or halofuginone, showed distinguished efficacy in removing *T. orientalis* infection. In India, Aparna *et al*. (2011) showed decrease in the prevalence of new infections after administration of buparvaquone.

2.7.2 Tick control

Once treated, animals become carriers and reservoir of the disease, thus a source of infection for new ticks that will feed on them. The main objective in the fight against theileriosis should be to prevent animals to become infected in the first place, through tick control (Office International des Épizooties [OIE], 2009). The most used tick control is the use of acaricides. Dipping or pour on throughout the year, mainly with flumetrin has shown to decrease the incidence of new infections in different countries (Shimizu, Nojiri, Matsunaga, Yamane & Minami, 2000; Yokoyama *et al*., 2011).

However this method requires a proper monitoring, and raises several issues such as the high price of acaricides, the increased tick resistance to these drugs, the environmental damage and the fact that, due to climate change, some species are appearing in areas where they had never been recorded before (OIE, 2009). Therefore, other methods for the decline of tick burden, such as rotational grazing, proper fencing of the herd, strict cattle movement laws or even the development of a vaccine against exposed tick antigen, are gaining ground (Sugimoto & Fujisaki, 2002).

2.7.3 Immunization

With respect to vaccination much remains to be done since there is no approved vaccine against *T. orientalis*. A live vaccine has been tested in Japan, but its use was prohibited, as it was proposed to transmit other blood-borne pathogens (Sugimoto & Fujisaki, 2002). The mix nature of the infection and the genetic diversity of the parasite make the development of an effective vaccine challenging (Jeong *et al*., 2009). Nowadays researchers are still trying to develop vaccines. Some studies are exploring a sporozoite surface antigen similar to P67 of *T. parva* or Su6-1 of *T. annulata* (Sugimoto & Fujisaki, 2002), since these two species have effective vaccination.

Also the major piroplasm surface protein (MPSP) has been studied for the development of a vaccine, given that the passive transfer of anti-MPSP mononuclear antibodies inhibits the progression of parasitemia (Boulter & Hall, 1999).

2.8 Detection of *T. orientalis* parasites

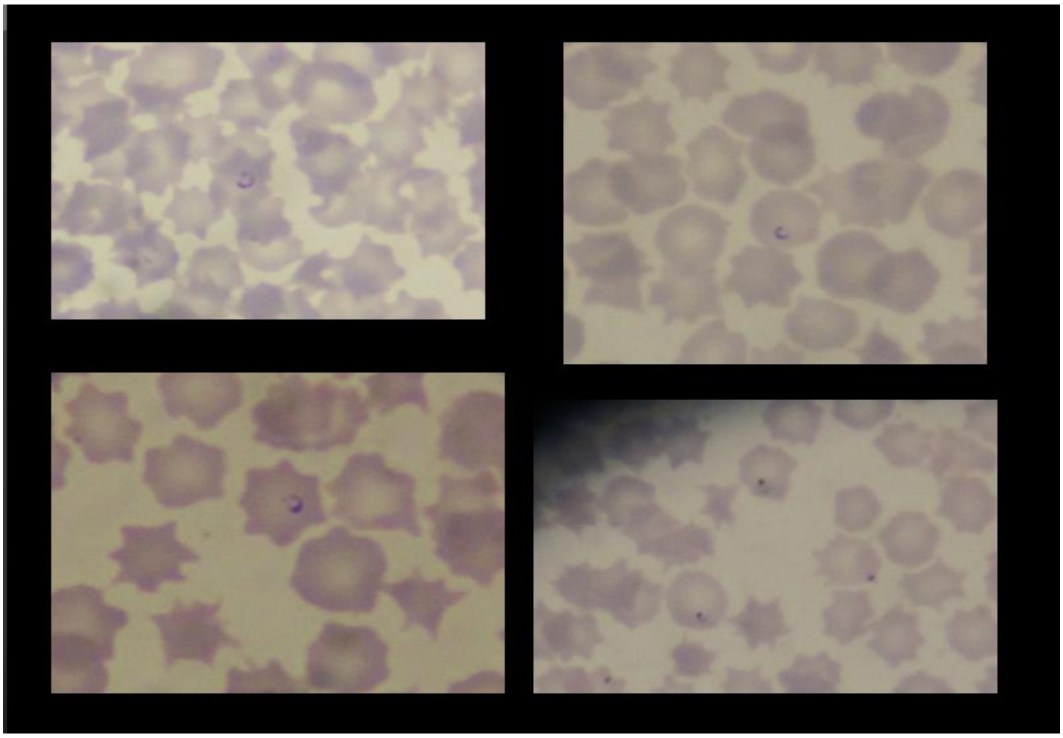
T. orientalis causes subclinical disease characterized by low growth rates and feed inefficiency (Jeong, Kweon, Kang & Paik, 2003). However acute syndromes may occur, where the animal shows pale or icteric mucous membranes, apathy, inappetence, with reports of abortion and still births in preparturient cattle (Izzo *et al*., 2010; Islam *et al*., 2011). After eliminating other plausible causes of haemolytic anaemia such as babesiosis, anaplasmosis, trypanosomosis, leptospirosis, Bovine Viral Disease (BVD), copper poisoning or preparturient hypophosphataemia (Aparna *et al*., 2011) a presumptive diagnosis of theileriosis is made. However, as this syndrome shows no pathognomonic signs, the confirmation of the parasite in the laboratory is a required demand for a final diagnosis.

2.8.1 Parasitological methods

Conventional diagnosis of theileriosis in Australia is by microscopic observation of the parasite on Giemsa-stained blood smears, where it appears as round, oval, pyriform, comma or rod-shaped basophilic inclusions in erythrocytes (Taylor *et al*. 2007) (Figure 14).

However, this approach requires expertise, does not always allow the diagnosis of carrier animals or those with low parasitemia and, in the presence of other parasites, such as *Babesia* or *Anaplasma* species, a misdiagnosis is readily possible (Kajiwara, Kirisawa, Onuma & Kawakami, 1990; Stewart *et al*., 1996; Liu *et al*., 2010).

Figure 14 –*Theileria* spp. in the red blood cells from cattle (original)



2.8.2 Serological methods

Another approach for diagnosis of *T. orientalis* infections is through the use of serological tests. These tests allow the identification of the parasite, by showing the presence of antigen-antibody reaction, and although largely used for epidemiological studies, they fail in the early detection of the parasite (Kajiwara *et al.,* 1990).

The indirect fluorescent antibody test (IFAt) detects the antigen-antibody reaction through immunofluorescent microscopic examination using ultraviolet light. The most common dyes/fluorochromes are fluorescein isothiocynate and rhodamine as they can be attached to the Fc region of the antibody without jeopardizing their specificity (Kuby, 1997). It is the most widely used serological test for East Coast Fever (*T. parva*) and tropical theileriosis (*T. annulata*) (OIE, 2009). Regarding *T. orientalis* it was used in the past for epidemiological surveys (Stewart, Standfast, Baldock, Reid & de Vos, 1992; Papadopoulos, Brossard & Perie, 1996) and it was considered to be highly specific under Australian conditions in the detection of *T. orientalis* parasites.

The World Organisation for Animal Health (OIE) (2009) considers the Enzyme-Linked Immunosorbent Assay (ELISA) test easy to interpret, more robust for field and more specific than IFAT. ELISA is based on the principle that an enzyme can be used to detect an antibody-antigen reaction, using an enzymatic colour reaction (Kuby, 1997).

Indirect ELISA were developed for *T. parva* and *T. mutans*, based on recombinant antigens, and showed higher sensitivity, detecting antibodies for a longer period of time when compared to IFAT (OIE 2009). Kawazu *et al*. (1992) used the ELISA test to compare different stocks of *T. sergenti, T. orientalis* and *T. buffeli,* and recently Wang *et al.* (2010) developed an indirect method, employing recombinant piroplasm protein.

Although these tests are fairly specific and easy to perform, their sensitivity depends on the course of infection, since the antibodies titres of carrier animals decrease as the disease progresses, which means that after some time, these animals can show a negative result and, in regions where different *Theileria* spp. co-occur it is difficult to achieve a reliable diagnosis, as cross reactivity commonly occurs.

2.8.3 Polymerase Chain reaction (PCR) – based methods

As serological and immunological (phenetic) methods are often not sufficiently specific and sensitive, particularly for animals with low parasitaemia, and/or in geographical regions where more than one strain/species of parasite is present, molecular tools have better characteristics for the diagnosis of infection/s. In addition some of these tools are also useful for epidemiological and population genetic studies, which is an advantage when studying a species whose classification is still controversial at this stage. PCR-based methods are widely used, and they have the potential to detect the presence of a single organism by specific amplification of its DNA.

The polymerase chain reaction (PCR) can be described as the "art" of replicate DNA in vitro. This method enables the amplification of a target zone in the gene in study by hybridization of a pair of oligonucleotides probes which serve as primers for the DNA synthesis. PCR occurs in a number of denaturation, annealing and extension cycles, resulting in millions of molecules which represent the final amplicons (Brown, 2010).

In theory, any sequence from any DNA can be amplified by PCR as long as specific primers are designed.

The amount of DNA to be amplified can be greatly reduced since just one double-strand is needed, in the first cycle, to hybridize with the primers, for the process to begin. After each cycle, the started amount is amplified by a factor of 2 (Nelson, Lehninger & Cox, 2008). It is important to note that this process is very sensitive, so any sequence of DNA that pair with the primers will potentially be amplified. That said the contamination leads to erroneous result. So it is important to work carefully and to ensure that reagents are free of nucleic acids, DNA-ases and RNA-ases. Critical is the use of a positive control (a sample already known to amplify) and a negative control (without template). If the positive control reveals a negative, then PCR steps should be optimized and the concentration of the reagents carefully studied. If, on the other hand, the negative control is positive, then the PCR was contaminated with erroneous DNA, and this problem should be addressed.

The main constituents/parameters needed for a PCR reaction are:

1. Primers: the key to the specificity of the PCR, since they determine the region to be amplified. They should be complementary to the region to be analysed and the 3' end should point towards to each other and must not hybridize between them. As to its length, must be neither too short so they won't hybridize with non-specific regions, nor too long, as the hybridization goes at slower rates, which culminates in the production of small quantities of product.

2. DNA polymerase: there are multiple choices of DNA polymerases but for PCR usually Taq polymerase is used as it is thermo stable, and therefore it resists the denaturation temperatures. This enzyme is isolated from *Thermus aquaticus,* a bacterium that lives in hot springs. It should be used in a concentration of 0.5 – 1.25U/ 50µl of PCR reaction, as low amounts may lead to less amount of product, and excessive amounts may lead to misincorporation of nucleotides, when associated to long extension timings ;

3. Deoxyribonucleotide triphosphate (dNTP): equal amounts of deoxyadenine triphosphate adenine (dATP), deoxythymidine triphosphate (dTTP), deoxycitosine triphosphate (dCTP) and deoxyguanidine triphosphate (dGTP), should be used in the reaction as they will be incorporated in the new strand. If one or more of the dNTPs is in higher concentrations than the remaining, misincorporations can occur, which will decrease the fidelity of the method; its stated that 200µl of each dNTP is sufficient in a 50µl PCR reaction;

4. Magnesium chloride: it is present in the reaction for delivery of magnesium, a co-factor of Taq polymerase;

5. Buffer: Tris-HCl provides an optimal pH, 8.3–8.8 at 20 ºC, for the PCR reaction;

6. Temperatures: denaturation – usually temperatures of 94-95 ºC are used in this step, which allows the breakage on hydrogen bonds of the double stranded DNA, and doesn't affect the polymerase activity. It also depends on the sequence content of C and G, since the higher their concentration is more hydrogen bonds are present, higher temperatures are needed to break them; annealing – the most important step as it can affect the specificity of the method. Usually temperatures ranging 50 to 72 ºC are used. The important is that the annealing temperature is not that high that will not allow the hybridization to occur, and not too low that will give raise to mis-hybridization and misincorporation. Normally this temperature is set in

agreement with the melting temperature (Tm) of primer-template (1-2 degrees below); extension – the optimal temperature for Taq activity is 72 ºC.

Using optimal primers, PCR has proved to be highly sensitive and specific for the detection of *T. orientalis* infections (Tanaka *et al.*, 1993; d'Oliveira, van der Weide, Habela, Jacquiet & Jongejan, 1995; Kawazu, Kamio, Sekizaki & Fujisaki, 1995).

Following PCR-amplification, products are analysed using the agarose gel electrophoresis. This technique is performed by running a portion of the PCR product in an agarose gel, dipped in buffer that will allow the electrical flow to be carried out from the negative to the positive pole. This way the amplicons will migrate through the gel and therefore they will be separated on basis on their molecular weight. A DNA leather of known size is used to help the characterization of the PCR products. After electrophoresis the agarose gel is stained and photographed.

2.8.3.1 Target region

Multiple DNA markers have been used for the detection and epidemiological surveys of *T. orientalis* populations.

The major piroplasm surface protein (MPSP) gene is the most studied genetic marker for *T. orientalis* (Gubbels *et al*., 2000). It is a single copy gene, conserved among all *Theileria* spp. and encodes an immunodominant protein in the piroplasm surface expressed during its intraerithrocytic stage (Kubota *et al*., 1996). It has a molecular weight that ranges from 32 to 34 kDa (Matsuba *et al*., 1995) and it is believed to have an important role in the pathogenesis of the parasite by modulating the host’s immune response (Jeong, 2010).

The analysis of the small subunit ribosomal RNA (SSU rRNA) gene have proven useful for the classification of different *Theileria* spp. (Allsopp, 1993). This multiple copy gene has been used in many studies and typing of the V4 variable loop region revealed 7 different genotypes for the organisms in the *T. orientalis* group: types A to E, H and Warwick (Chae *et al.*, 1998). A study by Gubbels *et al.* (2000) showed good correlation between MPSP and SSU classification within the same isolate.

Recently studies of the internal transcribed spacers (ITS 1, ITS 2 and the 5.8S rRNA gene) were carried out in order to discriminate between different species of *Theileria* (Aktas *et al*., 2007; Bendele, 2004).

2.8.3.2 Restriction Fragment Length Polymorphism (RFLP)

Restriction endonucleases are used to cut the PCR product in specific regions, as they recognise specific sequences within the amplified region. Therefore, different sequences will be cut in different regions, resulting in different patterns. After digestion, the product is run on agarose gel and photographed after staining.

2.8.3.3 Reverse Line Blot hybridization (RLB)

The RLB method allows the simultaneous identification of an onset of samples, obtained by PCR, through the use of specific probes. In this technique a first PCR amplification is undertaken using primers that will amplify all genotypes/species, after which the PCR amplicons are loaded in the RLB membrane and hybridization with species/genotypes specific probes occurs. The reaction is then revealed through a radioactivity or luminescent reaction.

2.8.3.4 Single-Strand Conformation Polymorphism (SSCP)

SSCP is a method to directly analyse the genetic variation present in PCR products, based on the principle that in solution single-stranded DNA adopts secondary and tertiary conformations, which means that a difference in a single nucleotide will result in a different conformation (Gasser *et al*., 2007). Thus, different propagation mobility, thereby a different pattern will show up in the gel. This means that SSCP technique separates different sequences not only based on their length but as well as on their physical and chemical characteristics.

Several studies have successfully employed SSCP to discriminate variation within and among PCR products, in parasites (Gasser & Monti, 1997; Gasser & Chilton 2001), as well as in protozoan organisms (Gasser *et al*., 2001; Gasser *et al*., 2003; Nolan, Jex, Mansell, Browning & Gasser, 2009).

2.8.3.5 DNA Sequencing

The most widely method used is the chain termination method invented by Fred Sanger (1981). In this method, a PCR-like reaction occurs with a few differences. In the chain termination method, there is the synthesis of new DNA molecules, but unlike the PCR reaction, here, only one primer is used. This serves as a rail that will help the DNA polymerase to start the inclusion of the different nucleotides. However a major difference, the key to success of the method, relies in the fact that, besides the dNTPs used in a typical PCR reaction, ddNTPs are also included, ie dideoxynucleotides (nucleotides to which the 3' end lacks the hydroxyl group.) As the syntheses goes, when the DNA polymerase includes a ddNTP the reaction stops, because in the absence of the hydroxyl group, the next nucleotide cannot be incorporated, since it lacks this group to create a phosphodiester bond. This leads to the production of different chains of various sizes, which will depend on the site where the ddNTP is incorporated. Finally the product is loaded in a polyacrylamide gel and electrophoresis is carried out, separating molecules according to their length. As each ddNTP corresponds a colour (for example green is ddATP, red is ddTTP, black is DDGTP and blue is ddCTP) when read in fluorescence detector it is possible to know which colour/nucleotide is present in a given position.

2.9 Final remarks

Theileria species are important apicomplexan parasites affecting mainly domestic and wild ungulates throughout the world. *T. parva*, *T. annulata* and *T. orientalis*, are highly significant pathogens and cause major economic losses mainly in Africa, Asia and Europe, but not in Australasia.

This literature review has shown that *T. orientalis* can represent a serious threat to cattle in parts of Australia. Despite being considered benign, recent reports show that theileriosis due to *T. orientalis* group is spreading to the southern parts of this country and demonstrate that this parasite is capable of causing serious disease in periparturient cattle, characterized by abortions, anaemia and depression, which at all resembles clinical cases observed in Japan and Korea, where the parasite is responsible for severe syndromes.

Despite the advancement of science and development of molecular-diagnostic tools, scientists have not reached an agreement on the taxonomy, classification and number of species that compose this group. Moreover, there is a huge gap in the knowledge of the interactions between parasite and mammalian host, as many aspects of pathogenesis and epidemiology are unclear. For example, although it is undisputed that this species cause disease, particularly through the development of haemolytic anaemia, the mechanisms by which it develops remain unknown, as well as the animal's immune response to this parasite remains unclear; therefore it is not understood whether seroconversion or cross-immunity occurs between/among different genotypes/species. Moreover, the epidemiology of the disease is still unclear in different states of Australia. For instance there are no recent studies of the prevalence and dynamics of infection in different parts of the country. The main vector of this disease is believed to be a tick of the genus *Haemaphysalys*: however, there are no recent studies to establish whether there are other means of transmission.

Importantly, there is no effective treatment or vaccine against *Theileria*, although buparvaquone has been demonstrated to be efficacious against theileriosis in cattle, this drug is not registered for the use in cattle in Australia, such that prevention and control rely on a sound understanding of the epidemiology of the disease, which is not achievable without the use of molecular tools.

CHAPTER 3 – GENETIC ANALYSIS OF THEILERIA ORIENTALIS POPULATIONS IN CATTLE FOLLOWING A THEILERIOSIS OUTBREAK IN VICTORIA, AUSTRALIA

3.1 Research aims

The present study addresses some of the gaps in our knowledge of *T. onrientalis* infections in Victoria. The specific aims of the study are:

1. Use of a molecular tool to study the prevalence of different genotypes of *Theileria* on a farm, in Victoria following a theileriosis outbreak in cattle;
2. Access the level of genetic variation within *T. orientalis* population;
3. Evaluate the utility of a molecular method for use as a diagnostic and analytical tool for the monitoring and surveillance of theileriosis in Australia and other countries;

3.2 Characterization of the "problem" farm

The farm affected by the theileriosis outbreak, is located in Seymour, 2km south of Tallarook in the southern state of Victoria, Australia (37° 07′ N, 145° 05′ E) (Figure 15). The property is almost isolated, having a country road on the north side, the freeway on the east side and the railway on the opposite side of the former.

The herd consists on average of 152 animals, in a 850 acres extension land, of which two are males and the remaining, females (cows and heifers), divided into two groups: one group consisting in one Shorthorn bull and Shorthorn cows, and a second group of one Charolais bull put together with Angus or cross bred AngusXCharolais cows. The groups are kept in separated paddocks, (keeping contact with each other through the wires) all year long (during summer temperatures can reach 40º C, and drop to 0º C during winter. Besides that rain fall is mainly winter and autumn but some also in the spring and is about 23 inches, average, so the owner doesn't have the need for stabulating the animals).

It is strictly a grazing property, in which animals are fed with hay, usually in large rolls in late summer and in winter, when feed is scarce in the paddocks.

For their breeding herd, they do not buy females in any breeders, just bulls and there are two birth seasons, one in the spring and another during autumn, so that animals can have an increased availability of pasture when their energy needs are at the highest peak. When calves from the breeding herd are about ten months old, they usually go to public sales yards.

Cattle are vaccinated as calves with 5 agents in one clostridial vaccine (against *Clostridium perfringens* type D - Pulpy Kidney; *C. septicum* - Malignant Oedema; *C. tetani* - Tetanus; *C. novyi* - Black Disease and *C. chauvoei* - Blackleg), receive a booster one month after the original and another when weaned if they are kept as replacements .

There are no cattle adjoining the property, only some horses on the southern border. But they use part of the property for a holdover for bullocks before going to slaughter. A load of two year old bullocks (steers) will come in on trucks usually from the north of the state and sometimes from New South Wales, put in the paddock for about one to two weeks at most and fed grain and hay in large feeders, then trucked to abattoir in Melbourne.

Figure 15 – Map of the Victoria state showing the localization of the farm suffering with the initial outbreak of theileriosis (adapted from http://en.wikipedia.org/wiki/File:Victoria_map.jpg)

3.3 Outbreak chronology

The Seymour vets were first contacted in 1st March 2011 to attend some shorthorn cattle that were aborting late term foetuses, about 7 months pregnant, and when the vets got there they were able to catch another animal in the paddocks, and manage to remove dead and smelly foetuses, around the same time of pregnancy as the other two.

As sick cows were showing signs of pronounced anaemia and jaundice and were with fever, some blood samples were taken and oxytetracycline was administrated.

In the next day the vets were called again to attend some new sick cattle, and more blood samples were taken.

The blood was tested for leptospirosis but the results came out low to negative for this disease, so they decided to investigate for the presence of *Theileria.*

Talking with the owners to get some more information, they found out that late in 2010, the farm received some cattle from NSW (a state where *T. orientalis* infection is endemic), and a short time after, some horses had a *H. longicornis* infestation (the tick vector for theileriosis in Australia).

At this point they made contact with Gasser's laboratory to help with their investigation and in a preliminary study, 15 blood samples from a herd of 60 cows plus one blood sample from one calf from the other herd were PCR tested.

When vets came back to the property they collected some more blood, and all tested positive for *Theileria orientalis*.

Three days later, at 10th March 2010, another cow died and one was aborting and before such a scenario, veterinarians and owners decided that the best thing to do would be treat all animals with large doses of oxytetracyclines to stop the infection to spread.

By the time this project was set to go, 4 cows and 2 calves died and a total of 8 still births counted for economic losses in the property.

In a way to better understand the dynamic of the outbreak, a chronologic table is presented below.

Table 3 – Detailed chronology of the Theileriosis outbreak

Date	Event
20/9/10 to 27/9/10	Approximately 118 cattle from NSW arrived to the property and stood there for one week
8/10/10 to 3/12/10	Approximately 50 steers were introduced into the farm
6/12/10	3 sick horses treated to bush tick infestation
8/12/10 and 23/12/10	Sick horses treated again 8 new cattle introduced in the farm
20/1/11	Weaner vaccination with Piliguard® and 5in1
28/1/11	Pregnancy test to autumn calving cows Some cows moved from autumn to spring herd 20 cows and 22 calves introduced in the spring herd
2/2/11	Sick horses treated
8/2/11	44 cattle sold
26/2/11	Dead cow and still born calf
1/3/11	Dead cow and still born calf – first veterinarian visit
2/3/11	Dead cow and still born calf Vaccination: 7in1, autumn cows and spring calving herd; 5in1 autumn weaners
7/3/11 8/3/11	Drenched all animals Administration of Alamycin LA 300®
10/3/11	Aborting cow
22/3/11	3 cows aborted late term
2/4/11	Cow delivered dead full term twin calves
6/4/11	Horse tested negative for *Theileria*
3/5/11	Dead cow and full term calf

3.4 Collection of blood samples

A total of 94 blood samples were collected by a qualified veterinarian from symptomatic and asymptomatic cattle from the affected farm. In addition, 12 and 10 samples were collected from asymptomatic cattle from two neighbouring, but unaffected, farms (2-5 km from the affected farm) (Table 4).

Once in the laboratory, 200µl aliquots, from each blood sample, were frozen at -80ºC.

Genomic DNAs were extracted from individual, whole blood samples using the DNeasy kit® (Qiagen, USA) according to the manufacturer's protocol.

The concentration of gDNA was then read in Nanodrop 100®, and samples were stored at -20C.

3.5 Enzymatic amplification

A portion of the MPSP locus (344 bp) was amplified by PCR from genomic DNA (~20 ng template) using newly designed primers (MPSP-AJ-F, 5′-TTCACTCCAACAGTCGCCCACA-3′; MPSP-AJ-R1, 5′-ACGTAAACTTTGACTGCGGTG-3′). PCRs were conducted in a 50 µl volume containing 10 mM of Tris-HCl (pH 8.4), 50 mM of KCl (Promega, USA), 3.5 mM of MgCl2, 200 µM of each of deoxynucleotide triphosphate (dNTP), 5 pmol of each primer and 1 U of GoTaq polymerase® (Promega). The PCR cycling conditions were: 95°C for 5 minutes (initial denaturation), followed by 35 cycles of 95°C for 15 seconds (denaturation), 60°C for 30 seconds (annealing), 72°C for 30 seconds (extension), followed by a final extension of 72°Cfor 10 minutes. For each set of PCRs, negative (no-DNA) and known positive controls were included. Following PCR, 5 µl of each amplicon were examined on 1.5% w/v agarose gels, which were stained with ethidium bromide for 20 minutes, destained in water for 2 minutes and then photographed (GelDoc, BioRad®) (figure 16). The remaining PCR products were stored at -20C.

Table 4- Representative table of blood samples collection from different farms. Farm 1 represents the farm suffering with theileriosis outbreak, and farms 2 and 3 the adjoining farms.

<table>
<tr><th>Farm</th><th>Date of collection (batch n)</th><th>Number of samples</th><th></th><th colspan="2">History</th></tr>
<tr><td rowspan="7">1</td><td rowspan="3">March 2011
#1</td><td>1 to 11</td><td rowspan="3">Adult Charolais</td><td colspan="2">Different herd from the original infected one
1 yearling died 1 week ago and found positive for Theileria sp</td></tr>
<tr><td>12 to 14</td><td rowspan="2">Original infected herd
Positive for Theileria sp two weeks ago</td><td>Aborted and were recovering</td></tr>
<tr><td>15 to 17</td><td>Were still pregnant</td></tr>
<tr><td>May 2011
#2</td><td>17</td><td rowspan="2">Shorthorn
Black baldy</td><td colspan="2">Original infected herd
Autumn calving group
Mixed of empty, aborted, calved and yet to calve animals</td></tr>
<tr><td>May 2011
#3</td><td>30</td><td colspan="2">Some calved and show no signs of disease
Some tested positive for Theileria sp prior</td></tr>
<tr><td>May 2011
#4</td><td>20</td><td rowspan="2">Angus
Charolais</td><td colspan="2">Animals from mob 2
19 out of 20 animals tested positive for Theileria orientalis prior</td></tr>
<tr><td>August 2011
#5</td><td>11</td><td colspan="2">Animals from mob 2</td></tr>
<tr><td>2</td><td>August 2011
#6</td><td>12</td><td>Adult Angus</td><td colspan="2">Property directly next to 1</td></tr>
<tr><td>3</td><td>August 2011
#7</td><td>10</td><td>Adult Hereford</td><td colspan="2">Property next to the farm 1 on Hume and Howell Rd</td></tr>
<tr><td></td><td>Total</td><td>116</td><td></td><td colspan="2"></td></tr>
</table>

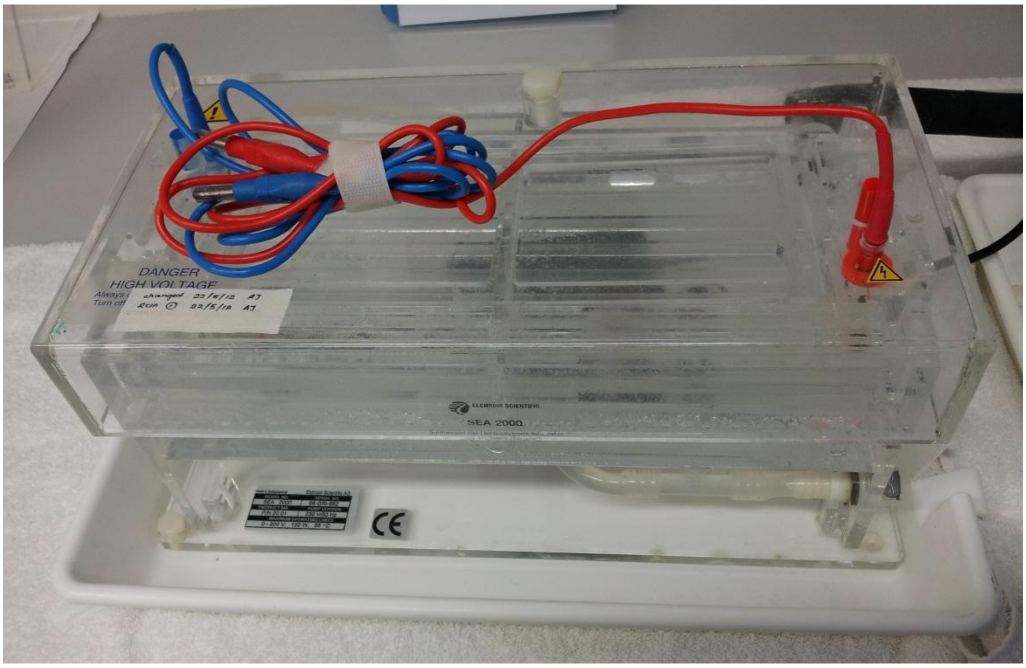
Figure 16 – Geldoc system (original)

3.6 Single-strand conformation polymorphism analysis

Single-strand conformation (SSCP) analysis (Gasser, Hu et al. 2007) was performed to display sequence variation within and among amplicons.

Briefly, 1-2 µl of each amplicon was mixed with 4 µl of H2O and 5 µl of sequencing stop-solution (cat. no. Q408A, Promega), heat-denatured at 94˚C for 30 min and then snap-cooled on a freeze block (-20 ˚C). Individual amplicons (7µl) were then loaded into the wells of precast GMA™ S-2x25 gels (Elchrom Scientific AG, Switzerland) and subjected to electrophoresis for 18 h at 74 V and 7.4 ˚C (constant) in a horizontal SEA2000™ apparatus (Elchrom Scientific) (Figure 17). Following electrophoresis, gels were separated from the plastic sheet with nylon line and stained for 30 minutes with SYBR Gold (Invitrogen, USA), destained in H20 for 15 min and photographed (GelDoc, BioRad).

Figure 17 – SSCP rig apparatus (original)



3.7 Sequencing

Amplicons (n = 3-5) representing each unique SSCP profile were selected, treated with shrimp alkaline phosphatase and exonuclease I (Fermentas Inc., USA) , and then subjected to bi-directional, automated sequencing (BigDye® Terminator v.3.1, Applied Biosystems, USA) using, separately, the same primers employed in PCR reaction. If extensive sequence ambiguity or polymorphism was detected, individual double-stranded DNA bands were excised from SSCP gels, DNA purified (QIAquick Gel Extraction Kit®, Quiagen) according to the manufacturer's protocol, and then PCR-amplified and sequenced (Gasser, Hu et al. 2007). The quality of each, forward and reverse, sequence was evaluated by appraising its electropherogram using the program BioEdit.

3.8 Phylogenetic analyses

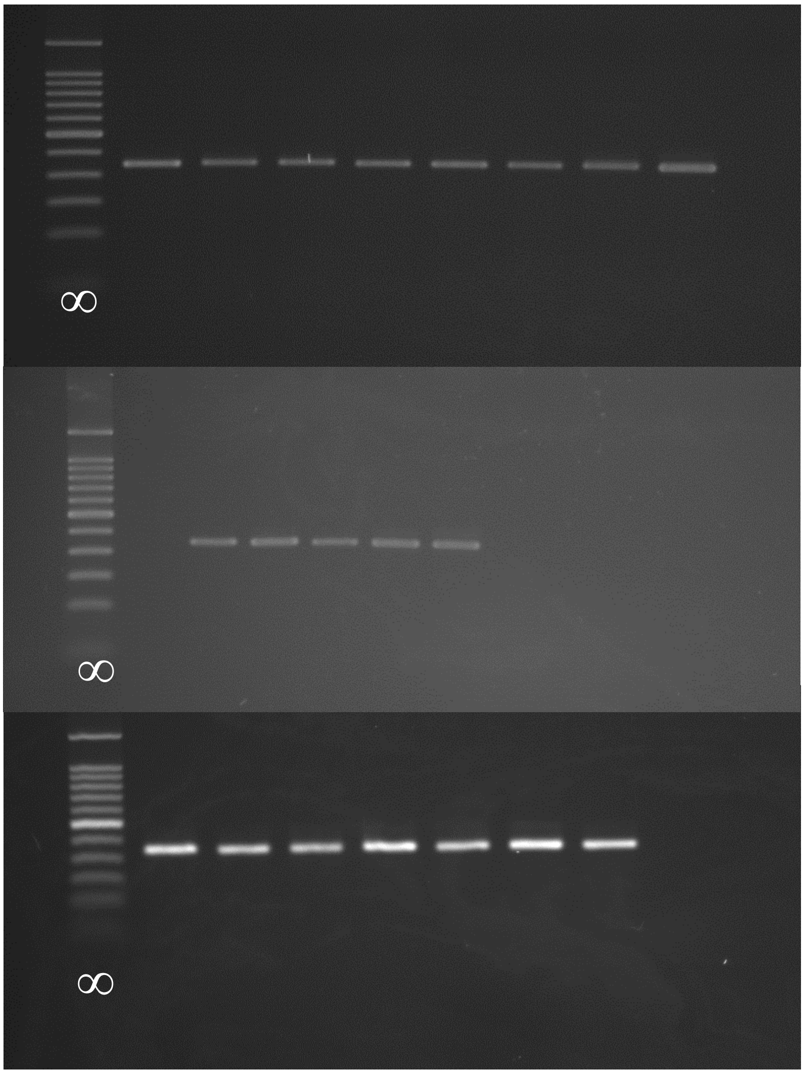
These analyses of sequence data (aligned over 301 homologous nucleotide positions) were conducted by Bayesian inference (BI), using the Monte Carlo Markov Chain (MCMC) method of MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) and the distance-based neighbour-joining (NJ) method. For BI, the likelihood parameters were set according to the Akaike Information Criteria (AIC) test in Modeltest v3.7 (Posada and Crandall 1998). The general time-reversible model of evolution, with gamma-distribution and a proportion of invariable sites, was utilised for the analysis of the sequence data. The estimates of base frequencies, the substitution rate model matrix and the proportion of invariable sites were fixed. Posterior probabilities (pp) were calculated for 2,000,000 generations, utilising four simultaneous tree-building chains, with every 100th tree being saved. At this point, the potential scale reduction factor (PSRF) approached one, and the standard deviation of split frequencies was <0.01. A consensus tree (50% majority rule) was constructed based on the final 75% of trees generated by BI. Sequence data were also subjected to analysis using the NJ method employing PAUP (PAUP4.0b10); distance was estimated employing the general time-reversible model of evolution, and the nodes were tested for robustness using 100,000 bootstrap replicates (Felsenstein 1985). Phylogenetic trees constructed using the two methods were examined for concordance in topology.

CHAPTER 4 – RESULTS AND GENERAL DISCUSSION

4.1 Amplification and mutation scanning of blood samples

Amplicons of the expected size (344 bp) (Figure 18) were produced from 84 of 94 (89.4%) samples from cattle from the theileriosis-affected farm in Seymour; no amplicons were produced from any of the 22 DNA samples from cattle from the two unaffected, neighbouring farms. Using the present protocol, no amplicons were produced from bovine DNA (data not shown).

Figure 18 – Representative agarose gels of the MPSP amplicons (~344 bp) derived from cattle blood samples ∞ = 100 bp ladder.

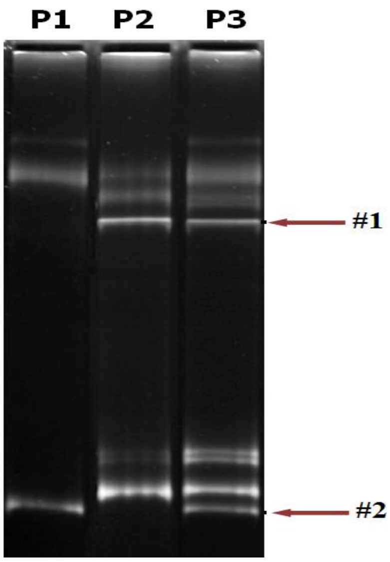


SSCP analysis of individual aliquots of all 84 amplicons displayed three distinct profiles: P1 (n= 63), P2 (n= 4) and P3 (n= 17) (Table 5). Profile P3 appeared to be a combination of profiles P1 and P2 (Figure 19).

Table 5 – Summary of SSCP results for MPSP locus.

Batch	PCR Pos / neg	SSCP profile		
		P1 (n)	P2 (n)	P3 (n)
#1	15 /1	13	1	1
#2	17 / 0	12	2	2
#3	29 /1	19	1	10
#4	20 / 0	19		1
#5	3/11			3
		63	4	17

Figure 19 – Representative SSCP gel displaying profiles P1, P2 and P3, and the bands (#1 and #2) which were excised from the SSCP gel for subsequent PCR-coupled sequencing



Selective sequencing showed that profiles P1 and P2 represented sequence type Vic-I (GenBank accession no. JQ781070) and sequence type Vic-C (GenBank accession no. JQ781071), respectively (Islam, Jabbar *et al.* 2011), which differed by 13.7% over an alignment length of 301 positions. As expected, sequences from individual amplicons representing profile P3 could not be unequivocally determined, indicating mixed sequence types or sequence heterogeneity.

In order to demonstrate that profile P3 represented a mix of profiles P1 and P2, two bands (#1 and #2; arrowed in Figure 19) in profile P3, which could be separated based on a sequence difference of ~41 bp, were excised from the SSCP gel and DNA purified using the Qiaquick gel extraction kit (QIagen,USA) according to manufacturer' protocol. DNA was then diluted (1/50), PCR-amplified and sequenced directly using the same primers as for PCR. Bands #1 and #2 were shown to represent profiles and sequence types Vic-C and Vic-I (Islam, Jabbar et al. 2011), respectively, proving that profile P3 was indeed a mix of profiles P1 and P2. Therefore, by inference, all 17 samples displaying profile P3 in SSCP analysis represented mixed populations of *T. orientalis*.

4.2 Phylogenetic analysis

The sequence types Vic-I and Vic-C (over 301 bp of the MPSP locus) were aligned along with homologous sequences representing all currently recognized genotypes within the *T. orientalis* complex, and representative sequences for *T. annulata* and *T. parva* (outgroups) available from the GenBank database. Phylogenetic analyses of these sequence data showed that all samples with sequence types Vic-I (n = 63) and Vic-C (n = 4) grouped, with maximum statistical support, with sequences representing the ikeda and chitose genotypes of *T. orientalis*, respectively, to the exclusion of all other currently recognized *T. orientalis* genotypes (Figure 20). The topology of the trees constructed using the two different algorithms was the same, with some variation in nodal/bootstrap support values.

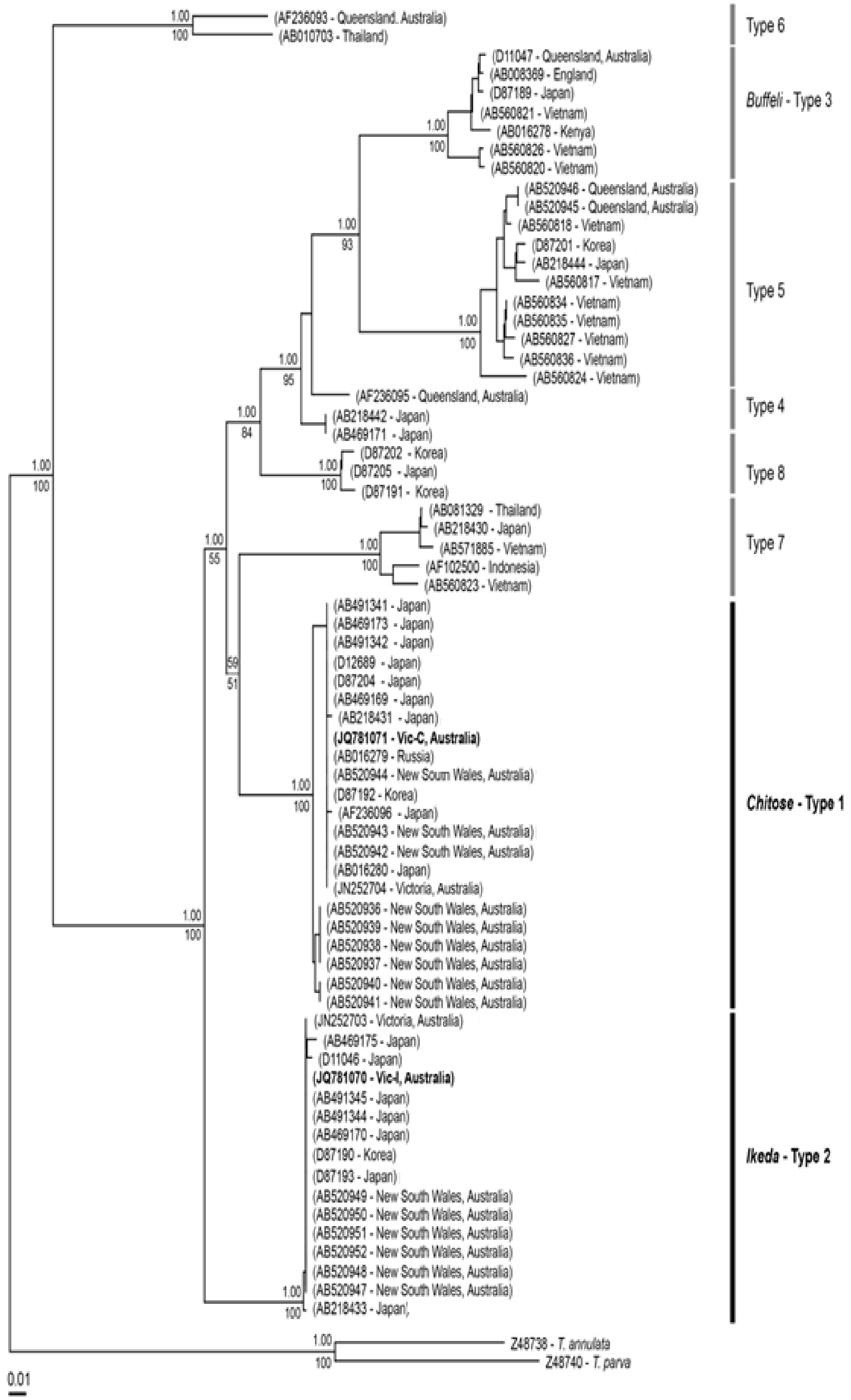


Figure 20 – Relationships of partial *MPSP* nucleotide sequences from *Theileria* from cattle in Victoria (present study; bold-type) and those representing all currently recognized genotypes (“*types*” *1-8*). Accession numbers of sequences and geographical origins are given in round brackets.

4.3 General discussion

The present mutation scanning-based analysis detected *T. orientalis* infection in 84 samples out of 116 tested. Using a mutation-scanning method was also possible to discriminate between the *ikeda* and *chitose* genotypes in 75% and 4.8% of total infected cattle, respectively, and mixed populations of these two genotypes in 20.2% of infected cattle.

Although this is the first detailed genetic analysis of *T. orientalis* linked to bovine theileriosis in the state of Victoria, there is unpublished evidence of at least 51 other outbreaks on beef or dairy cattle farms in other, disparate regions of this state (personal communication with Dr. Michael Jeffers, Department of Primary Industries, Victoria; January 2012). The sharp increase in the number of outbreaks in Victoria (from six to 51 during 2011) raises substantial concerns that theileriosis could have a major economic impact on farms, at least in the short term.

Current evidence indicates that either or both of the genotypes (i.e. *ikeda* and/or *chitose*) detected here are usually associated with clinical theileriosis in cattle in the Asia Pacific region (Ota *et al*., 2009; Izzo *et al*., 2010; Islam *et al*., 2011; Kamau *et al*., 2011; McFadden *et al*., 2011). Furthermore, as discussed previously in the Literature Review chapter these are the predominant genotypes in Japan, Korea and China where oriental theileriosis causes major economic losses in livestock production. One way to reassure that the presence of these genotypes is the real and only cause for the outbreak would be by linking the severity of symptoms to different strains, *i.e.*, if the presence of ikeda and/or chitose infection would always manifest on the form of the disease or whether there are asymptomatic animals.

It is known that outbreaks occur especially when naive animals are exposed to the parasite, either by cattle movement to endemic areas, or by the introduction of infected ticks into clean areas. The present investigation shows that DNA of *T. orientalis* was detected in nearly 90% of cattle on a beef cattle farm near Seymour, even though only a small number of potentially *Theileria*-infected or tick-infested cattle were introduced from NSW (Islam *et al*., 2011). Currently, the reason/s for the rapid increase in the number of bovine theileriosis outbreaks in Victoria are unclear (Kamau *et al*., 2011) and deserves urgent exploration. Ticks of the genus *Haemaphysalis* occur in various areas of this state (Riek, 1982). However, it is not yet clear whether they are susceptible to infection with the genotypes of *T. orientalis* detected here and whether they would act as active intermediate hosts. Moreover, it is not yet known whether bovine theileriosis in Victoria is spreading *via* a species of *Haemaphysalis* or whether other vectors or alternative transmission routes might be possible (by mechanical transmission or iatrogenic infection).

Currently, no studies in Australia have yet investigated, in detail, sero-conversion/-reversion times in local cattle or whether immune responses are protective. Although Gale, Leatch, Dimmock, & Gartside (1997) showed that natural infected animals when challenge with *Anaplasma* spp. develop low parasitemia levels compared with free- *Theileria* animals, it is not known whether cattle exposed or infected with one particular genotype (e.g., *ikeda*) will mount a protective immune response against an infection with another genotype (e.g., *chitose*) of *T. orientalis*, and *vice versa*. Thus Matsuba *et al.* (1993) and Kubota *et al*. (1996) showed changes in the dominant population during chronic infection or during transmission from cattle to vector or vector to cattle, what may show a way of the parasite to evade and disrupt the immune system of the host, which may interfere with the assembly of a lasting and proper immune response. These questions should be addressed using accurate molecular and immunological tools. For example, the realization of a temporal study would be important. In this, different animals, carrying different genotypes and types of infection, would be tested in predetermined dates, to follow the evolution of the disease. It would also be interesting to test calves from infected mothers, short after birth.

Although this method proved to be more effective than the conventional diagnosis carried out in Australia (by the observation of blood smears, since it has the advantage, upon the latter, of detecting mixed infections as well as discriminate between different genotypes), it would have been interesting to test both the sensibility (for example through the comparison of results between different blood smears and PCR of the same sample) and specificity of the primers used here, through its use in samples previously genotyped.

Although the assignment of genotypes employing our mutation scanning-based sequencing and phylogenetic approach is rapid and practical, in the future, it will be important to undertake whole-genome sequencing using next-generation technology (e.g., Illumina) (Mardis, 2008) to enable the definitive, genetic identification of isolates and detailed comparative genetic analyses of key members within the *T. orientalis* complex. Such a genomic sequencing effort will allow the definition of a wide range of genetic markers of utility for population genetic studies as well as for the monitoring of genetic drift or shift in populations of the *T. orientalis* complex in Australia and elsewhere.

Particularly surprising and concerning has been the fact that no effective anti-theilerial compound (such as buparvaquone) (Minami *et al*., 1985; McHardy, 1989) is yet readily available in Australia to treat clinically affected cattle, thus exacerbating economic losses on farms with acute outbreaks. Although treatment trials with buparvaquone have been conducted (Carter, 2011), they have not yet been published in the peer-reviewed literature. Thus, current treatment options against theileriosis in Australia are very limited: registered drugs include oxytetracycline and imidocarb. To date, veterinarians in the states of NSW and Victoria (http://www.dpi.nsw.gov.au/_data/assets/pdf_file/0003/404679/Bovine-anaemia-caused-by-Theileria-orientalis-group.pdf; http://www.dpi.vic.gov.au/agriculture/ pests-diseases-and-weeds/animal-diseases/vetsource/benign-theileriosis) have reported variable responses to treatment with oxytetracycline, being 'good' for mildly affected animals, and 'poor' for severely affected cattle (The Department of Primary Industries Victoria and New South Wales, 2012). Current peer-reviewed information indicates that tetracyclines, being schizont-suppressive agents (Dolan, 1981; Singh, Gill, Kwatra & Sharma, 1993), are not as effective as other specific anti-theilerial drugs, such buparvaquone (Singh *et al*., 1993), which likely explains the variable treatment efficacy to oxytetracycline in affected cattle in Australia. Clearly, urgent attention needs to focus on enhanced treatment, diagnostic and control strategies. Importantly, an enhanced understanding of the molecular genetics, epidemiology and biology of members of the *T. orientalis* complex will be essential to underpin effective diagnosis, surveillance and control of bovine theileriosis in Victoria and other parts of Australia.

Altough a lot was acomplished with the project, as we proved that more pathogenic strains have entered the State, alerting for their economic impact, many questions remain without answer. The reason why there is a shift from an endemic status to an outbreak is still unknown: is it due only to the present of *Ikeda* and *Chitose* strains, or there are other unknown parcels in the equation; in this study no *Bufelli* parasites were found in the tested samples: were they replaced by other genotypes, have they remained in the field, it is unknown.

For these reasons, it would be important in the future undertake different epidemiological studies, in different, or even in all states of the country, in order to access the real prevalence of the parasite, using the method developed here as it have shown, to date, to be the faster and more discriminating.

Also it would be productive studing the buffaloes' subpopulation, in an area where the prevalence of *T. orientalis* is higher, since its role as a reservoir of this disease has not been effectively refuted or proved and, as it happens for other *Theileria*, may be an important key in the dynamic of the disease.

CHPATER 5 - CONCLUSION

The present thesis used a PCR coupled SSCP method to access and evaluate the prevalence, as well as the genetic variation of a *T. orientalis* population present in a farm involved in the first reported theileriosis outbreak in the southern state of Victoria, Australia.

This method allowed the detection of parasite DNA in almost 90% of samples, which were then genetically characterized by single-strand conformation polymorphism, in 3 different profiles: Ikeda (75%), Chitose (4.8%) and a third profile comprising both strains (20.2%). Despite the fact that the assignment of genotypes employing our mutation scanning-based, sequencing and phylogenetic approach is rapid and practical, in the future, it will be important to undertake whole-genome sequencing using next-generation technology (e.g., Illumina) to enable the definitive, genetic identification of isolates and detailed comparative genetic analyses of key members within the *T. orientalis* group. Such a genomic sequencing effort will allow the definition of a wide range of genetic markers of utility for population genetic studies as well as for the monitoring of genetic drift or shift in populations of the *T. orientalis* group in Australia and elsewhere.

Although this was the first prevalence study in a Victorian farm, a several number of other outbreaks (n = 51) have been reported from beef and dairy cattle throughout the state, which may indicate that the more pathogenic genotypes present in this study may be spreading at an alarming rate in southern parts of Australia.

In conclusion, although this study was an important step in the understanding of the theileriosis outbreak in this farm, by showing the prevalence of the two more pathogenic strains, there is still much more to be done with respect to the present knowledge of the disease. The method used here can now be expanded and optimized to detect remaining genotypes in order to be used as main tool for generating data for diagnosis, epidemiological and phylogenetic studies, as well as to provide a better insight on the treatment and control methods.

CHAPTER 6 – REFERENCES

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